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RESEARCH SECTION

SHORT PAPERS

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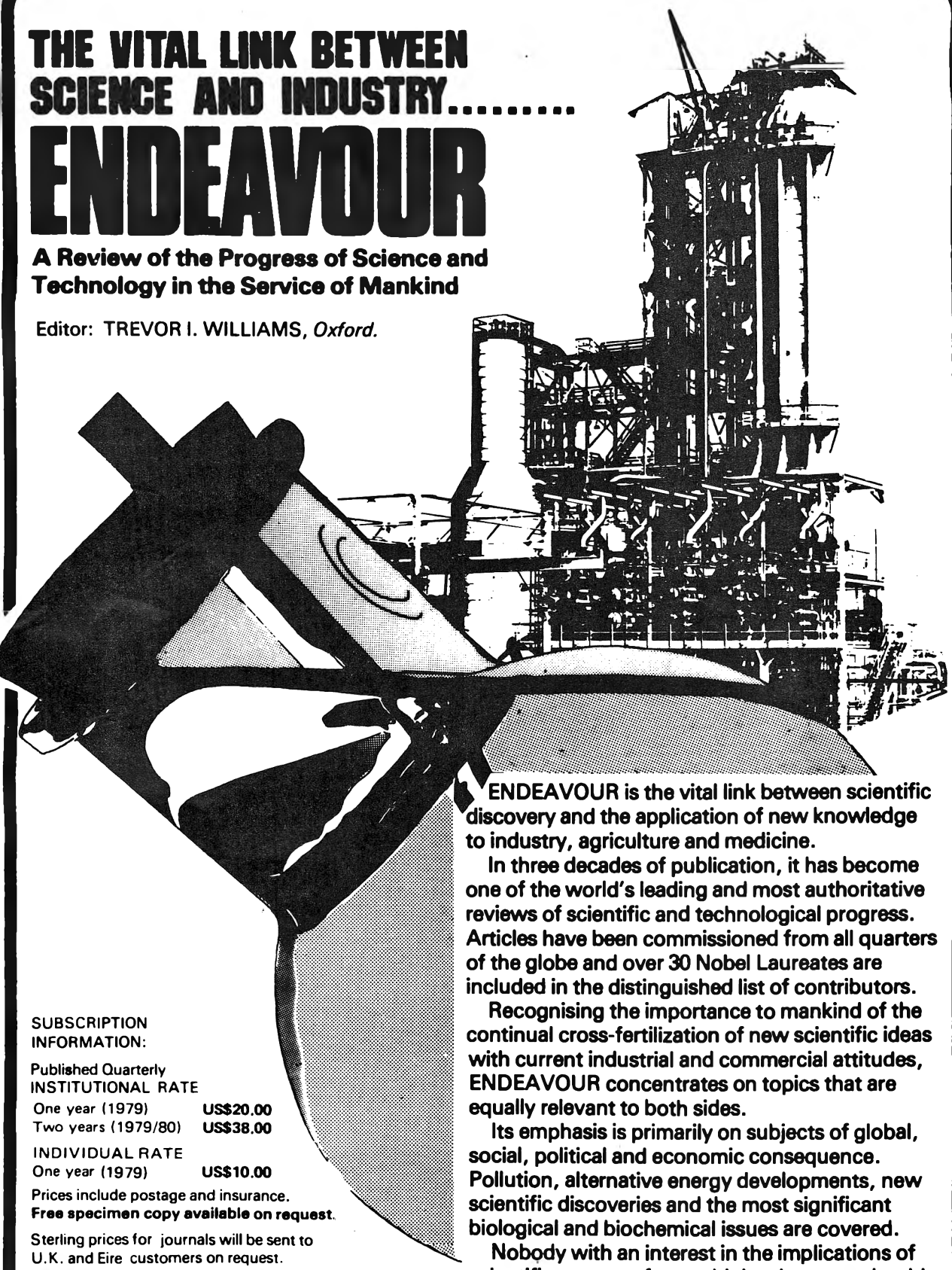

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Research Section

2-BROMO-2-NITROPROPANE-1,3-DIOL AS A NITROSATING AGENT FOR DIETHANOLAMINE: A MODEL STUDY

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(*Received 12 October 1978*)

Abstract—In a model study, 2-bromo-2-nitropropane-1,3-diol (BNPD), an antibacterial and antifungal agent, was shown to nitrosate diethanolamine and triethanolamine. The reaction between diethanolamine and BNPD in aqueous solution was studied in some detail and was found to be pH- and time-dependent, nitrosation being most effective when the initial pH was 12·0, as in an aqueous equimolar solution of BNPD and the amine. At this pH, the yield of *N*-nitrosodiethanolamine (NDELA) increased from $<1\%$ after 1 hr to a maximum of 11% after 96 hr. Yields of the nitrosamine declined significantly when the initial pH was lower. Decomposition products of BNPD in alkaline solution include nitrite, bromide, 2-bromo-2-nitroethanol, 2-nitroethanol, formaldehyde, 2-hydroxymethyl-2-nitropropane-1,3-diol and other compounds. On the basis of the data obtained, a mechanism is proposed by which BNPD releases nitrite ion, the assumed nitrosating species. BNPD is thus a nitrosating agent which is most effective in alkaline media.

INTRODUCTION

N-Nitrosodiethanolamine (NDELA) has been identified by Schmeltz, Abidi & Hoffmann (1977) in tobacco treated with certain formulations of the herbicide maleic hydrazide (MH-30). It has also been found in industrial cutting fluids (Fan, Morrison, Rounbehler, Ross, Fine, Miles & Sen, 1977b; Zingmark & Rappe, 1977) and in consumer products such as cosmetics, lotions and shampoos (Fan, Goff, Song, Fine, Arsenault & Biemann, 1977a). The presence of this carcinogenic nitrosamine (Druckrey, Preussmann, Ivankovic & Schmähl, 1967; Hilfrich, Schmeltz & Hoffmann, 1978) was attributed to the use of di- or triethanolamine in the above products, and to the amine's subsequent nitrosation. In tobacco, nitrite present in the plant (Tso, 1972) seems likely to be the agent responsible for the nitrosation. Nitrite has also been shown to occur in certain synthetic metal-cutting fluids along with di- or triethanolamine (Fan *et al.* 1977b). In these fluids, the latter amine undergoes nitrosation during storage, even at alkaline pH (Zingmark & Rappe, 1977).

2-Bromo-2-nitropropane-1,3-diol (BNPD) is an antimicrobial agent used in cosmetics and similar products, particularly because of its high activity against Gram-negative bacteria (Bryce & Smart, 1965). A previous study demonstrated that in tetrahydrofuran at 70° certain characteristic *C*-nitro compounds such as BNPD are capable of nitrosating morpholine (Fan, Vita & Fine, 1978). In such a system, BNPD effected a 0·2% nitrosation of morpholine.

The present study was undertaken to investigate the manner in which BNPD nitrosates amines of the type commonly found in cosmetic formulations, e.g. diethanolamine. As the subsequent discussion will show, BNPD effects the nitrosation of this amine in aqueous solution at ambient temperature in significant yield (11% after 96 hr). The parameters (time and pH) that influence the extent of the nitrosation reaction will be discussed and a mechanism by which nitrite is released from BNPD will be proposed. This nitrite is assumed to be the actual nitrosating species, and when present with certain amines in consumer products is apparently responsible for the *N*-nitrosamines reported therein.

EXPERIMENTAL

Chemicals. The organic solvents used were spectrograde and the other chemicals were reagent grade. Reagents for the Griess reaction (for nitrite determinations) and for the Volhard determination (for bromide) were obtained from Fisher Scientific Co., Pittsburgh, PA. Chromotropic acid, for the determination of formaldehyde, was obtained from Aldrich Chemical Co., Inc., Milwaukee, WI, and trimethylsilylating agents (Regisil) from Regis Chemical Co., Morton Grove, IL. BNPD and diethanolamine were purchased from Aldrich Chemical Co. and triethanolamine from Fisher Scientific Co. Purities of these materials were $>98\%$ as determined by chromatographic techniques. 2-Nitroethanol was obtained from Aldrich Chemical Co. and 2-hydroxymethyl-2-nitropropane-1,3-diol from Pfaltz and Bauer, Inc., Stamford, CT. The latter material required purification by thin-layer chromatography (TLC) prior to its use as a reference. 2-Bromo-2-nitroethanol was synthesized by the method of Gold, Hamel & Klager (1957). The synthesis and properties of *N*-nitrosodiethanolamine (NDELA) have also been described (Schmeltz *et al.* 1977).

Apparatus. High-pressure liquid chromatography (HPLC) was performed on a Waters Associates Model GPC-204 instrument equipped with a 6000 A

solvent delivery system, a U6K septumless injector, a 660 solvent programmer, and a 440 UV/visible detector. Chromatographic separations were achieved on a microporasil column (30 cm × 3·9 mm). Gas chromatography (GC) was run on a Hewlett-Packard Model 7620A instrument equipped with a flame ionization detector, and gas chromatography–mass spectroscopic analyses (GC–MS) on a Hewlett-Packard system consisting of a 5980A mass spectrometer interfaced with a 5710 gas chromatograph and a 5933A data system. For GC analysis, a 1·8 m × 0·32 cm stainless-steel column packed with 3% SE-30 on 80/100 mesh Gas Chrom Q was used. A Bausch and Lomb Spectronic 20 colorimeter was used for determination of nitrite following the Griess reaction (520 nm), and of formaldehyde following reaction with chromotropic acid (570 nm).

Procedures

Reaction of BNPD with diethanolamine. Equimolar amounts of diethanolamine (0·53 g) and BNPD (1·14 g) were added to 2–5 ml H_2O at ambient temperature. The pH of this solution was 11·5–12·0. After 15 min, the solution turned yellow and gradually darkened, until after 6 hr it turned a deep red. At specified times, an aliquot (1–5 μl) was injected into the HPLC apparatus. The microporasil column was washed with a solution of 4% ethanol in chloroform at a flow rate of 2·5 ml/min. BNPD eluted at 3·8 min and NDELA at 6·0 min. The limits of detection for BNPD and NDELA were, respectively, < 3 μg and < 15 ng/μl of injection. Peaks were triangulated and compared to those from standards for purposes of quantitation. The pH of other reaction mixtures was lowered with HCl, at the start, to determine the effect of pH on the course of the reaction.

Reaction of BNPD with triethanolamine. The reaction between BNPD (1·14 g) and triethanolamine (0·75 g) was carried out and monitored as above.

Analysis of products. NDELA and BNPD were determined by HPLC as described above. Also used was trimethylsilylation followed by GC, as described by Schmeltz *et al.* (1977). Identification of the reaction products was confirmed by mass spectral and chromatographic comparison with reference compounds (NDELA, 2-nitroethanol and 2-nitro-2-bromoethanol).

The degradation products of BNPD in solution at various pH values were monitored for bromide and nitrite. BNPD (230 mg) was dissolved in 1–2 ml H_2O and the pH was adjusted to 12·0, 8·0 or 3·0 by careful addition of either 2 N-NaOH or 0·5 N-H_2SO_4. These solutions were analysed at given intervals for nitrite by a modification of the Griess reaction (Furman, 1962), after adjustment of the volume to 25 ml with water, and for bromide by the Volhard titrimetric method (Furman, 1962), after adjustment of the solution volume to 5 ml and acidification with dilute (1:1) HNO_3 (detection limits < 1 and < 500 ppm, respectively).

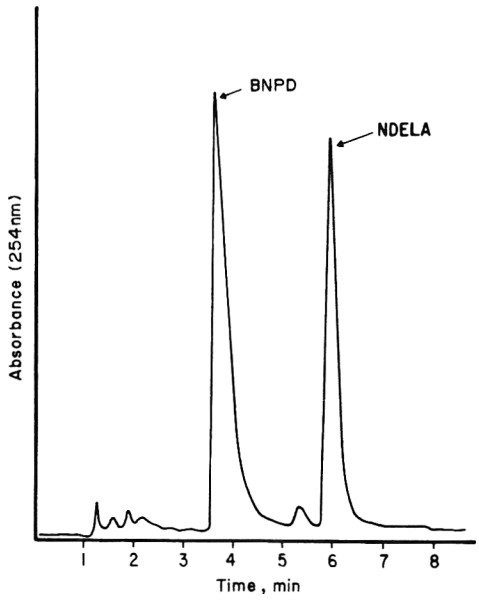

Fig. 1. HPLC trace of the reaction mixture of BNPD and diethanolamine at 24 hr.

Formaldehyde was determined colorimetrically (570 nm) by reaction with chromotropic acid (Patai, 1966; detection limit < 1 ppm).

RESULTS

HPLC in conjunction with a UV detector was used to follow the degradation of BNPD on reaction with diethanolamine, and the formation of NDELA (Fig. 1). More peaks were evident early in the reaction, including those of 2-nitroethanol and 2-nitro-2-bromoethanol, both of which eluted well before BNPD and NDELA. The effluent corresponding to a prominent peak emerging between BNPD and NDELA was apparently unstable. On collection, it decomposed into NDELA and other compounds, as seen on reinjection into the chromatograph. The peak in question also decreased in size and eventually disappeared during the course of the reaction, and was not seen at all in solutions of BNPD made alkaline with NaOH rather than with diethanolamine. It is evident that under these conditions instability is a feature not

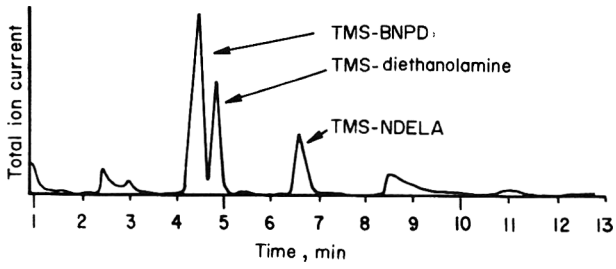

Fig. 2. Gas chromatogram of trimethylsilylated (TMS) reaction mixture of BNPD and diethanolamine.

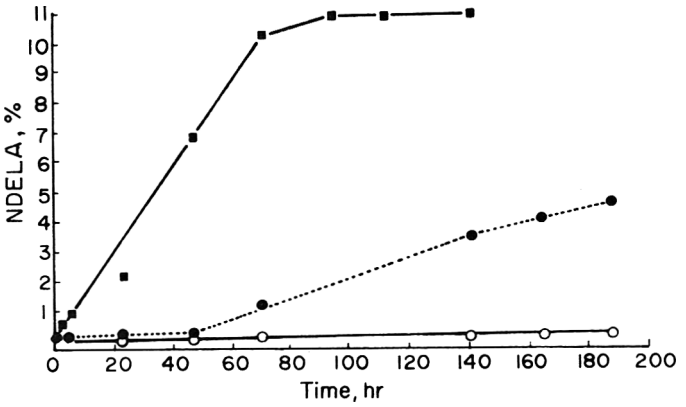

Fig. 3. Formation of NDELA in aqueous equimolar solutions of diethanolamine and BNPD as a function of time at pH 4·0 (○), 8·0 (●) and 12·0 (■).

only of BNPD but also of some of its degradation and reaction products. One such reported product, 2-hydroxymethyl-2-nitropropane-1,3-diol (Bryce, Croshaw, Hall, Holland & Lessel, 1978) did not produce a peak in an HPLC unit equipped with a UV detector, nor was it demonstrated by GC of the trimethylsilylated products of the BNPD–diethanolamine reaction. BNPD and NDELA, however, were easily detected in reaction mixtures by GC of their trimethylsilyl derivatives. In this case, a peak attributable to diethanolamine was also seen (Fig. 2). GC–MS confirmed the identities of these materials.

Mixing equimolar amounts (5 mM) of BNPD and diethanolamine in water (5 ml) produced a solution of pH 12·0. Under these conditions, levels of NDELA increased gradually, reaching 11% after 96 hr (Fig. 3), and 30% of the initial BNPD was fairly rapidly degraded. Lowering the initial pH of the BNPD–diethanolamine solution reduced the yields of NDELA. Nitrosation by BNPD obviously occurred more readily at higher pH. After about 70 hr the rate of formation of NDELA began to slow down. This decrease in rate may have been related to the gradual lowering of the pH of the reaction mixture with time (Table 1).

Like the formation of NDELA in BNPD–diethanolamine solutions, the formation of nitrite and bromide in alkaline solutions of BNPD is time- and pH-dependent. The formation of these ions in BNPD solutions at different pH values was measured in the absence of diethanolamine because the orange to reddish colouration resulting from the reaction between BNPD and diethanolamine interferes with the determinations. The degradation of BNPD in solutions made alkaline with NaOH was assumed to be analogous to its degradation in solutions made alkaline with amine. (Bromide, nitrite and formaldehyde were also identified in BNPD–diethanolamine solutions.) The rate of release of bromide and nitrite from BNPD at pH 12·0 was maximal within the first hour (Table 2; Figs 4 & 5). During this period, bromide formation was 100 times faster than that of nitrite. Nitrite concentrations may be higher in NaOH-adjusted than in amine solutions of BNPD because, in the latter

Table 1. *Formation of NDELA in equimolar aqueous solutions of BNPD and diethanolamine*

Time (hr)	pH*	NDELA formed	
		μg/litre	%†
0	12·0	—	—
1	8·5	425	0·06
3	8·3	4100	0·61
6	8·1	6740	1·00
24	6·0	14,800	2·21
48	5·5	46,500	6·94
72	5·4	69,100	10·3
96	5·4	73,500	11·0
114	—	73,800	11·0
144	—	74,500	11·1

*pH of the reaction mixture (containing initially 5 mmol of each reactant in 5 ml H_2O at pH 12) at the given time.

†Percentage of theoretical yield.

Table 2. *Bromide and nitrite levels in aqueous solutions of BNPD* as a function of time and pH*

Time (hr)	Concnt† (μM) of		Rates of formation (μmol/mmol BNPD/hr)	
	NO_2^-	Br^-	NO_2^-	Br^-
		pH 12·0		
0·5	0·8	80	1·4	139·2
1	5·1	120	4·4	104·3
3	6·3	140	1·8	40·6
6	7·4	200	1·1	29·0
24	10·7	200	0·4	7·2
48	12·2	200	0·2	3·6
		pH 8·0		
0·5	0·03	—	0·06	—
1	0·2	30	0·2	26·1
3	0·9	60	0·3	17·4
6	1·3	100	0·2	14·5
24	3·0	120	0·1	4·3
48	3·2	120	0·06	4·3
		pH 3·0		
0·5	—	—	—	—
1	—	—	—	—
3	0·01	—	0·003	—
6	0·01	—	0·003	—
24	0·02	—	0·001	—
48	0·02	—	0·001	—

*Initial concentration: 1·15 mM in 5 ml H_2O; pH adjusted with NaOH or H_2SO_4.

†Where no value is given, the amount was below the detection limit.

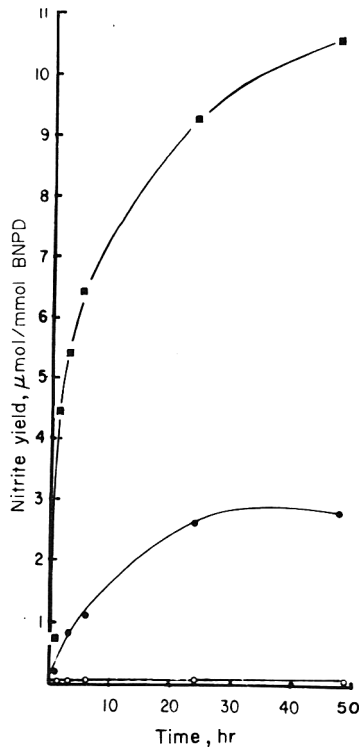

Fig. 4. Formation of nitrite in aqueous BNPD solutions at pH 3·0 (○), 8·0 (●) and 12·0 (■).

case, additional nitrite is consumed as a result of the nitrosation reaction.

As with NDELA, the formation of bromide and nitrite occurs most readily at high pH. It is for this reason, and because of the presence of formaldehyde in alkaline BNPD solutions (reported by Bryce & Smart (1965) and confirmed in this study), that diethanolamine (as well as triethanolamine; Table 3) undergoes the rather facile nitrosation observed in this work. At pH 12·0, the level of formaldehyde reached a plateau at 0·8% after 3 hr, at pH 8·0, it reached a level of 0·5% in the same period, and at pH 3·0, no formaldehyde was detected until after 24 hr (< 0·005%). BNPD is apparently fairly stable in an acid medium, undergoing virtually no degradation in concentrated sulphuric acid.

DISCUSSION

The results obtained demonstrate that the antimicrobial agent, BNPD, commonly used in cosmetic preparations, readily degrades to yield nitrite, especially at high pH. Diethanolamine, also present in certain cosmetic formulations, is easily nitrosated under these conditions, thus providing an explanation for the reported presence of NDELA in cosmetics and similar products. Formaldehyde, shown previously and in this study to be another degradation product of BNPD, would probably catalyse the nitrosation in alkaline medium (Keefer & Roller, 1973). The nitrosation is a slow reaction, and the level of NDELA reaches a maximum after about 96 hr, but some nitrosation occurs immediately. In NaOH solutions, BNPD degradation follows a course similar to that demonstrated in solutions of diethanolamine and

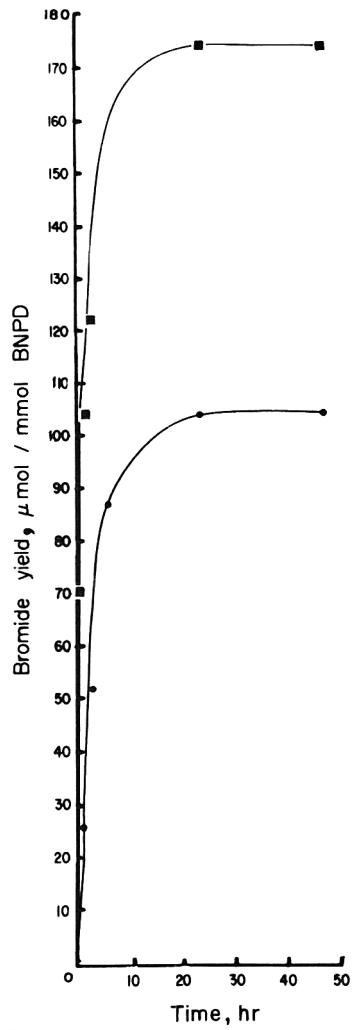

Fig. 5. Formation of bromide in aqueous BNPD solutions at pH 8·0 (●) and 12·0 (■).

addition of the amine results in the formation of NDELA. NDELA also formed in aqueous solutions of BNPD and triethanolamine, although in lower yields.

Since bromide ion is released from BNPD much more rapidly than is nitrite ion, one can speculate

Table 3. *Formation of NDELA in an aqueous solution* of BNPD and triethanolamine*

Time (hr)	NDELA formed†	
	μg/litre	%‡
0	—	—
1§	43·5	0·006
4	115	0·02
24	316	0·05

*Initial content of solution: 1·15 g BNPD and 0·75 g triethanolamine in 5 ml H_2O.

†No NDELA was found in control solutions of BNPD or triethanolamine alone (limit of detection by HPLC 15 ng).

‡Percentage of theoretical yield.

§Time of first measurement.

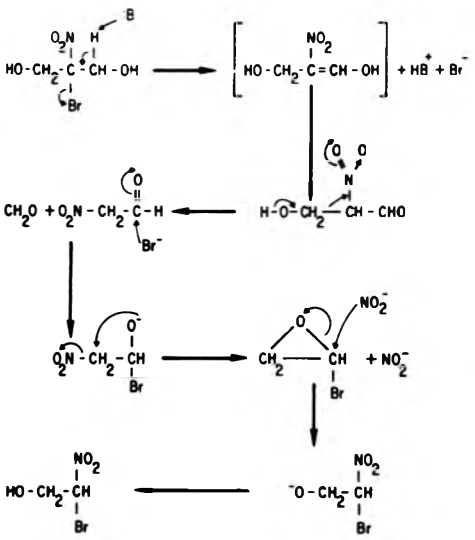

Fig. 6. Proposed pathway for the degradation of BNPD in alkaline medium (:B = base).

that the pathway of BNPD degradation in alkaline medium could be that indicated in Fig. 6. A base (B) attacks the acidic hydrogen alpha to the carbon bearing the nitro and bromo groups, with the resulting elimination of HBr. The pathway then proceeds as illustrated, with the subsequent release of formaldehyde and nitrite. Nitrite is then available for the nitrosation of any appropriate amine present, as well as for the formation of 2-nitro-2-bromoethanol. This pathway is somewhat different from that proposed by Bryce *et al.* (1978), who envisaged an initial release of formaldehyde rather than of bromide. However, our data indicate that the loss of bromide initiates the breakdown of BNPD, with subsequent release of formaldehyde and nitrite.

This study demonstrates the efficacy of BNPD as a nitrosating agent for both secondary and tertiary amines in alkaline solution. Consumer products that are prepared with this antimicrobial and alkanolamines may have a similar potential for generating *N*-nitrosamines. The need for further studies on such products of known composition is therefore indicated.

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IN VITRO METABOLISM OF [^{14}C]RUBRATOXIN B BY RAT HEPATIC SUBCELLULAR FRACTIONS

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(*Received 7 July 1978*)

Abstract—The metabolism of rubratoxin B was studied *in vitro* using rat hepatic subcellular fractions. Primarily, metabolism of rubratoxin B involved a non-enzymatic process in the microsomal supernatant fluid. The transformation products were mainly water-soluble, although at least four unidentified organosoluble derivatives of rubratoxin B were detected. Three of these organosoluble derivatives were produced in the microsomal supernatant fluid by an enzymatic process.

INTRODUCTION

Rubratoxin B (Fig. 1) is a hepatotoxic (Burnside, Sippel, Forgacs, Carll, Atwood & Doll, 1957) metabolite of the fungi *Penicillium rubrum* and *P. purpurogenum*. Other toxic effects attributed to this compound include mutagenicity (Evans & Harbison, 1977) and teratogenicity (Hood, Innes & Hayes, 1973). Congestive, haemorrhagic and degenerative changes of the liver and spleen were the most common histological lesions associated with rubratoxin B toxicity in a variety of animals (Wogan, Edwards & Newberne, 1971). The LD_{50} of rubratoxin B was 0·36 mg/kg when dissolved in propylene glycol and administered ip to adult rats, increasing to 400 mg/kg when given by stomach tube (Wogan *et al.* 1971). Hayes, Cain & Moore (1977), however, reported the oral LD_{50} of rubratoxin B in neonatal rats to be 6·34 mg/kg, indicating a 60-fold increase in the susceptibility of young animals. Pretreatment of adult male mice with SKF-525A resulted in a 60% decrease in the ip LD_{50} of rubratoxin B (Hayes & Ho, 1978).

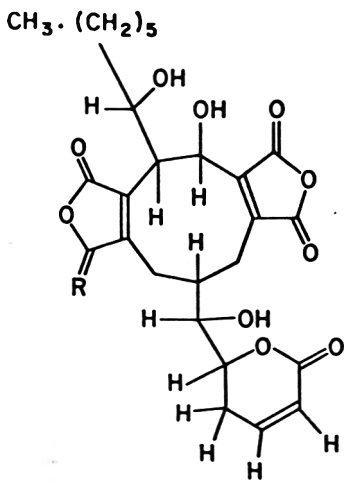

Fig. 1. Structure of the rubratoxins, rubratoxin A: $C_{26}H_{32}O_{11}$: R = H, OH. Rubratoxin B: $C_{26}H_{30}O_{11}$: R = 0. In aqueous solution the maleic anhydride moieties on either side of the nine-membered ring equilibrate with the open form to yield the carboxylic acid derivative.

All detectable radioactivity in the liver 30 min after ip administration of 0·05 mg/kg [^{14}C]rubratoxin B to male mice was found in the microsomal supernatant fluid (Hayes, 1972). Within the first 2 hr, increasing amounts of radioactivity were found in the mitochondria with a parallel decrease of radioactivity in the microsomal supernatant fluid. By 24 hr, the amount of total liver radioactivity in the microsomal supernatant fluid had decreased to 80% with less than 10% present in the microsomal fraction.

The effects of ip administration of 1·0 mg/kg rubratoxin B on the activities of the mouse liver microsomal enzymes nucleoside triphosphatase, hexobarbital hydroxylase, aminopyrine demethylase, *p*-nitroanisole-*o*-demethylase, aniline hydroxylase and NADPH oxidase have been examined (Hayes, 1977). The activities of nucleoside triphosphatase, hexobarbital hydroxylase and aniline hydroxylase remained unchanged after treatment of the animals with rubratoxin B. The activities of aminopyrine demethylase, *p*-nitroanisole-*o*-demethylase and NADPH oxidase, however, were decreased by 18, 58 and 70%, respectively.

Rubratoxin B, upon exposure to aqueous medium, is rapidly and reversibly converted to a carboxylic acid derivative which is far more polar than the parent compound, and is less toxic to mice (Unger, Phillips & Hayes, 1978). Hydrolysis of the maleic anhydride moieties on either side of the nine-membered ring results in the formation of the carboxylic acid derivative (Fig. 1). The carboxylic acid derivative is converted back to the parent form by acidification to pH 2 or below (Unger & Hayes, 1978).

EXPERIMENTAL

Animals. Male Sprague-Dawley rats (350–380 g) were kept in temperature-controlled animal facilities away from known hepatic enzyme inducers, on bedding made from corn cobs.

Chemicals. [^{14}C]Rubratoxin (36 μCi/mmol) used in these experiments was produced by surface cultures of *P. rubrum* (NRRL; A-11785) grown in liquid medium containing ^{14}C-labelled D-glucose (Hayes, 1972). The toxin was recovered from the growth

medium and purified by the method of Hayes & Wilson (1968). Identity and purity of the compound were established by melting point, IR spectroscopy, high-pressure liquid chromatography (HPLC) and mass-spectral analysis. Based on gas flow analysis of thin-layer plates used to purify rubratoxin, the toxin used in these studies was 98% radiopure. Glucose 6-phosphate (G6P), G6P-dehydrogenase and NADP were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were of analytical reagent grade.

Intracellular localization of rubratoxin B metabolism. Rats were killed by decapitation and livers weighing 13–15·5 g were immediately removed. The livers were washed with ice-cold 1·15% (w/v) KCl, cut into small pieces and homogenized in 3 vol 1·15% KCl using a Potter-Elvehjem-type homogenizer. The resulting suspension was filtered through fine-mesh nylon to remove fibrous debris.

The intracellular localization of rubratoxin B metabolism was established by assaying the various fractions obtained by differential centrifugation. The concentration of each preparation was adjusted to be equivalent to 0·25 g liver/ml. Protein content of each subcellular fraction was estimated by the method of Lowry *et al.* (1951).

Aliquots of the whole homogenate (39–45 mg protein/ml) were centrifuged at 10,000 **g** (max) for 30 min in a Sorvall RC-5 refrigerated centrifuge. The sediment (which contained cellular debris, nuclei and mitochondria) was resuspended in 1·15% KCl (w/v) and the final suspensions contained 19·5–21·5 mg protein/ml. Microsomal fractions were isolated from the post-mitochondrial supernatant fraction by centrifugation at 105,000 **g** (max) for 60 min using a Beckman type 50 fixed-angle rotor in a Beckman L5-65 ultracentrifuge. The supernatant fluids (containing 24·2–26·2 mg protein/ml) were decanted, and the microsomal suspensions contained 2·0–3·5 mg protein/ml.

Metabolism of rubratoxin B. Aliquots (1 ml) of each of the subcellular fractions were incubated with labelled rubratoxin B in a 2·5-ml reaction mixture containing phosphate buffer, pH 7·4 (56 mM), KCl (27·7 mM), $MgCl_2$ (5·0 mM), nicotinamide (10 mM), NADP (0·24 mM), G6P (4·0 mM) and G6P-dehydrogenase (1·0 IU). Aliquots of the whole homogenate were incubated with all but G6P-dehydrogenase. The [^{14}C]rubratoxin B was added in propylene glycol (10 μg/10 μl). The reaction was initiated by enzyme addition and during the 30-min incubation period, the open 25-ml Erlenmeyer flasks were shaken in a Dubnoff metabolic shaking incubator maintained at 37°C. The reactions were terminated by addition of 2·5 ml ice-cold methanol. Assays were also performed using heat-treated preparations (95°C for 30 min), and preparations lacking the NADP, G6P and G6P-dehydrogenase.

Rubratoxin B (10 μg) also was incubated in the complete incubation medium, in the incubation medium lacking NADP, G6P and G6P-dehydrogenase, and with nicotinamide alone (25 mM) to determine the interaction of the toxin with components of the incubation medium.

Extraction. The pH of aliquots (1 ml) of each mixture was adjusted to 2·0 with 0·5 N-HCl and the acidified mixture extracted twice with an equal volume of benzene. The defatted aqueous phase was then extracted twice with equal volumes of ethyl acetate. The ethyl acetate extracts were pooled, evaporated to dryness under vacuum, and the residue dissolved in acetonitrile for analysis by HPLC.

Analysis. Aliquots of each mixture were mixed with 10-ml Aquasol (New England Nuclear Corp., Boston, MA) and radioassayed in a Beckman LS-250 liquid scintillation spectrometer (Fullerton, CA). The aqueous phase from each extraction was mixed with 10-ml Aquasol and radio-assayed. Reverse-phase HPLC was performed as previously described using a high pressure liquid chromatograph from Waters Associates (Milford, MA), with detection at 254 nm (Unger & Hayes, 1978). The eluate from the chromatographic analysis of each mixture was collected at 10-sec intervals and radioassayed.

RESULTS

As shown in the HPLC chromatogram tracing in Fig. 2, rubratoxin B eluted as a sharp peak at 175 sec, corresponding to fractions 18–19 on the radioactivity profiles shown in Figs 3–8. The incubation of rubratoxin B in the incubation medium alone, with or without NADP, G6P and G6P-dehydrogenase, resulted in the conversion of the toxin to an unidentified compound (Table 1). Collection and radioassay of the eluate from the HPLC analysis indicated that this compound was more polar than the parent molecule, eluting mainly in fractions 11 and 12 (Product III, Fig. 3). As indicated in Table 1, after 30 min incubation at 37°C, only 34% of the rubratoxin B added to the complete incubation medium could be extracted as parent compound. When NADP, G6P and G6P-dehydrogenase were excluded from the incubation medium, 25% of the added rubratoxin B was recovered. In addition, a small amount of radioactivity was recovered from the aqueous phase.

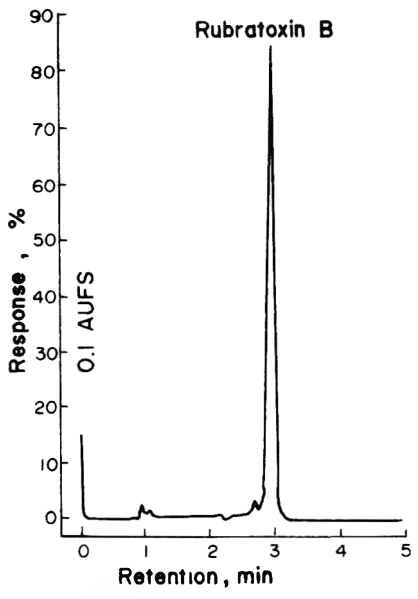

Fig. 2. Chromatogram tracing of rubratoxin B standard (1 μg injected, detection at 254 nm), at a sensitivity of 0·1 absorbance units full scale (AUFS).

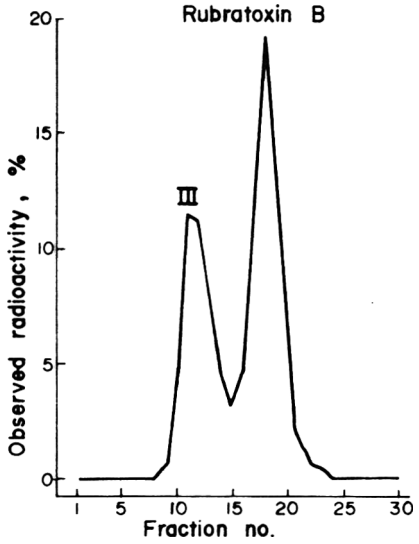

Fig. 3. Radioactivity profile of the eluate collected at 10-sec intervals from the HPLC analysis of the incubation medium after exposure to [^{14}C]rubratoxin B (10 μg) for 30 min at 37°C.

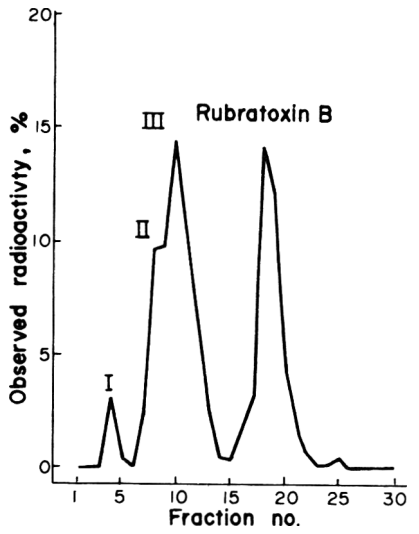

Fig. 5. Radioactivity profile of eluate collected at 10-sec intervals from the HPLC analysis of the 10,000 g × 30 min sediment after incubation with [^{14}C]rubratoxin B (10 μg) for 30 min at 37°C.

The data in Table 2 indicate that transformation of rubratoxin B occurred primarily in the microsomal supernatant fluid, only 1·83% of the initial dose of rubratoxin B remained as the parent compound after 30 min of incubation. Transformation by the microsomal supernatant fluid was mainly to water soluble compounds (78·48% of the initial dose) with about 20% being converted to organosoluble derivatives. Heat treatment of the microsomal supernatant fluid resulted in only a doubling of the amount of unchanged rubratoxin B (to about 3·6% of the initial dose), and had little effect on the production of water-soluble derivatives. Leaving the NADPH-generating system out of the incubation mixture, however, resulted in a five-fold increase in the amount of rubratoxin B remaining unchanged, decreased the production of water-soluble derivatives, and nearly doubled the production of non-water-soluble derivatives. Transformation of rubratoxin B by the microsomal preparation appeared to have been mainly due to the incubation medium. However, 11·82% of the initial dose of rubratoxin B was converted to water-soluble compounds by the microsomal preparation. Heat-treatment or incubation of the microsomal fraction in the absence of NADP, G6P and G6P-dehydrogenase resulted in nearly a doubling of the water-soluble radioactivity and a decrease in the formation of non-water-soluble derivatives. The use of heat-treated

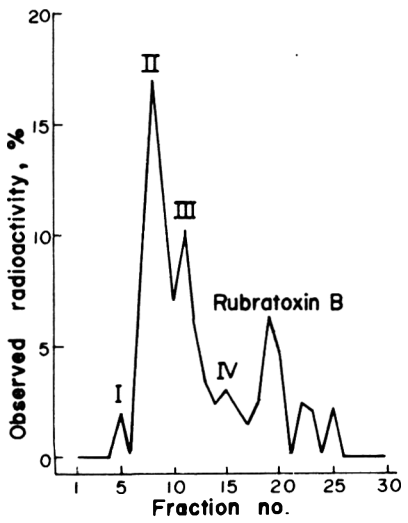

Fig. 4. Radioactivity profile of eluate collected at 10-sec intervals from the HPLC analysis of the whole homogenate after incubation with [^{14}C]rubratoxin B (10 μg) for 30 min at 37°C.

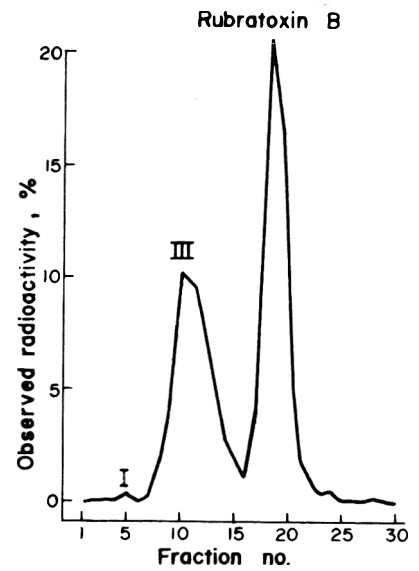

Fig. 6. Radioactivity profile of eluate collected at 10-sec intervals from the HPLC analysis of the 105,000 g × 60 min sediment after incubation with [^{14}C]rubratoxin B (10 μg) for 30 min at 37°C.

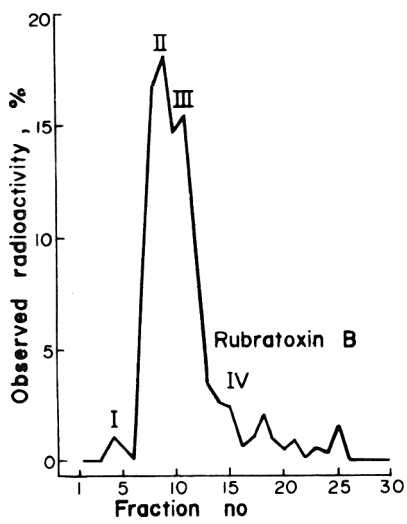

Fig. 7. Radioactivity profile of eluate collected at 10-sec intervals from the HPLC analysis of the 105,000 g × 60 min supernatant after incubation with [^{14}C]rubratoxin B (10 μg) for 30 min at 37°C.

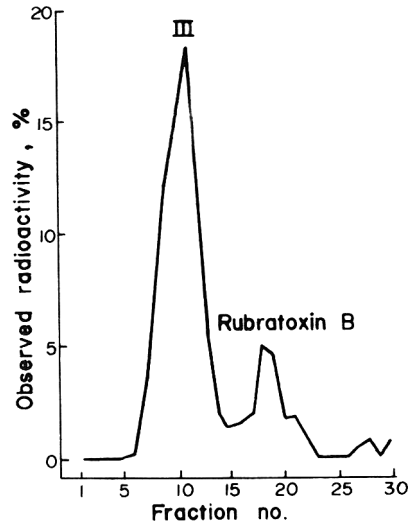

Fig. 8. Radioactivity profile of eluate collected at 10-sec intervals from the HPLC analysis of the 105,000 g × 60 min supernatant after the absence of an NADPH-generating system.

microsomal preparations or incubation in the absence of an NADPH-generating system resulted, therefore, in a shift of the pattern of conversion (towards water-soluble compounds) rather than a decrease in the amount of conversion, since there was no significant change in the amount of rubratoxin B left unchanged.

Incubation of rubratoxin B in the whole homogenate resulted in the formation of at least four unidentified organosoluble products (Fig. 4), eluting in fractions 4–5 (product I), 8–9 (product II), 10–11 (product III) and fraction 15 (product IV). In addition, two other peaks of radioactivity, less polar than the parent compound, eluted in fractions 21–22 and 24–25. Product III was formed when rubratoxin B was exposed to the incubation medium alone, in the absence of tissue (Fig. 3). Peaks corresponding to each of these could be found in one or another of the subcellular extracts. Products I, II and III were observed in the incubate of the sediment after centrifuging at 10,000 g for 30 min (Fig. 5), and products I and III in the microsomal incubate (Fig. 6). Products II and IV, however, appear to have been contributed mainly by the microsomal supernatant fraction (Fig. 7). When rubratoxin B was incubated with the microsomal supernatant fraction in the absence of an NADPH-generating system, products I, II and IV were not formed (Fig. 8). Neither were these products formed when rubratoxin B was incubated with the heat-treated microsomal supernatant.

DISCUSSION

The results of this investigation indicated that the transformation of rubratoxin B was most active in the microsomal supernatant fraction. The transformation was primarily non-enzymatic. However, in the microsomal supernatant, addition of a NADPH-generating system resulted in an increase in conversion of rubratoxin B to water-soluble derivatives. Products I, II and IV were not formed by the heat-treated microsomal supernatant or in the absence of NADPH, indicating enzymatic involvement. Also, maximum conversion of rubratoxin B to non-water-soluble derivatives by the microsomal fraction occurred in non-heat-treated preparations in the presence of NADPH.

Product III, appearing in fractions 10–11 of the whole homogenate radiochromatogram (Fig. 4), was probably non-biological in origin, since a corresponding peak was produced when rubratoxin B was exposed to the incubation mixture in the absence of

Table 1. *Interaction of rubratoxin B with incubation medium after 30 min at 37°C*

Incubation medium	Percentage of initial dose		
	Rubratoxin B*	Water-soluble† derivatives	Other derivatives‡
Whole incubation medium	33·86 ± 2·5	4·09 ± 0·6	62·05 ± 2·1
Incubation medium minus NADP, G6P and G6P-dehydrogenase	25·24 ± 1·7	1·18 ± 0·3	73·6 ± 2·1

*Determined by HPLC.
†Determined by radioassay of aqueous phase remaining from extractions.
‡Determined mathematically by subtracting from the initial dose the percentage dose determined for the parent compound and the percentage dose remaining in the aqueous phase.
Values are means ± SEM of triplicate incubations, each assayed in triplicate.

tissue (Fig. 3). This compound probably does not spontaneously form in biological fluids since it has not been detected in bile or plasma extracts from isolated perfused liver, and whole animal experiments performed in this laboratory.

Tables 1 and 2 reveal that most, if not all, of the transformation of rubratoxin B which occurred in the microsomal preparation could be accounted for as a non-biological conversion of the toxin to an unidentified organosoluble derivative (product III) with a retention time of about 120 sec (Figs 3 and 6). The requirements for this conversion appeared to be nicotinamide plus anion (phosphate or halide), since neither incubation with Ringer's solution (Unger & Hayes, 1978) nor nicotinamide (25 mM) alone resulted in the production of this derivative. Interaction of rubratoxin B with nicotinamide could have significant biological consequences, since nicotinamide is a precursor to vital co-factors (NAD, NADP).

Products I, II and IV were formed only in the presence of tissue fractions, and did not spontaneously form in the presence of the incubation medium alone. These products also were not formed by the microsomal supernatant fluid in the absence of NADPH, and were not formed by heat-treated microsomal supernatant fluid. The formation of these products therefore should be considered to be dependent on some enzymatic process. The formation of water-soluble derivatives of rubratoxin B also was seen to occur only in the presence of tissue fractions. Unlike organosoluble products I, II and IV, however, the formation of water-soluble derivatives also occurred using heat-treated fractions and in the absence of NADP, G6P and G6P-dehydrogenase. The maximum formation of water-soluble derivatives was dependent on some tissue factor present predominantly in the microsomal supernatant fluid, and to a lesser extent, in the sediment from the centrifugation at 10,000 g for 30 min. Little water-soluble radioactivity was found in the microsomal incubate.

Care must be taken in interpreting the results of this investigation. For example, it is unlikely that intracellular components would ever be exposed, as they are in this *in vitro* study, to appreciable concentrations of the carboxylic acid derivative of rubratoxin B. Previous studies indicated that high concentrations of rubratoxin B-derived radioactivity are not attained in the liver (Unger, Hayes & Mehendale, 1979). This suggests that rubratoxin B is probably absorbed as the parent compound, which represents a small percentage of the total rubratoxin present (Unger, Phillips & Hayes, 1978). Unaided transmembrane movement of the carboxylic acid derivative into the hepatocytes would be limited by its polarity and molecular size. It seems likely therefore that, in an intact system, subcellular components would be exposed to higher proportions of the parent compound relative to the carboxylic acid derivative.

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Table 2. *Intracellular localization and co-factor requirements of rubratoxin B metabolism*

Liver fraction	Percentage of initial dose		
	Rubratoxin B*	Water-soluble derivatives†	Other derivatives‡
Whole homogenate	1·95 ± 0·02(5·8)	73·14 ± 1·77(1788)	24·91 ± 1·77(40·1)
Heat-treated	6·31 ± 0·138(18·6)	71·06 ± 4·38(1737)	22·63 ± 4·74(36·5)
Minus NADPH-generating system	2·397 ± 0·1127(9·5)	71·61 ± 4·44(6069)	25·98 ± 4·47(35·3)
Sediment after centrifugation at 10,000 g for 30 min	14·02 ± 2·72(41·4)	55·13 ± 8·54(1348)	30·85 ± 5·83(49·7)
Heat-treated	10·98 ± 0·784(32·4)	63·87 ± 2·5(1562)	25·15 ± 3·7(40·5)
Minus NADPH-generating system	9·972 ± 0·441(29·5)	55·14 ± 3·39(4673)	34·885 ± 3·14(47·4)
Sediment after centrifugation at 105,000 g for 60 min	25·58 ± 1·75(75·5)	11·82 ± 2·05(289)	62·60 ± 3·89(101·8)
Heat-treated	26·59 ± 3·68(78·5)	18·17 ± 2·65(444)	55·24 ± 6·03(89·0)
Minus NADPH-generating system	21·62 ± 0·83(85·7)	18·55 ± 0·92(1572)	58·83 ± 0·62(80·1)
Supernatant after centrifugation at 105,000 g for 60 min	1·83 ± 0·04(5·4)	78·48 ± 1·51(1917)	19·69 ± 1·54(31·7)
Heat-treated	3·57 ± 0·56(10·5)	76·09 ± 1·36(1860)	20·34 ± 0·8(32·8)
Minus NADPH-generating system	9·29 ± 1·54(36·8)	56·63 ± 1·33(4799)	34·08 ± 2·52(46·3)

*Determined by HPLC.

†Determined by radioassay of aqueous phase of the extractions.

‡Determined mathematically by subtracting from the initial dose the percentage dose determined for the parent compound and the percentage dose remaining in the aqueous phase.

Values are mean ± SEM of triplicate incubations, each assayed in triplicate. The values in parentheses are the percentages of the control value. The control for the liver fraction and the heat-treated fraction was the whole incubation medium (Table 1), and the control for the liver fraction minus NADPH-generating system was the incubation medium minus NADP, G6P and G6P-dehydrogenase.

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THE DETERMINATION OF RESPIRABLE PARTICLES IN TALCUM POWDER

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Abstract—The mass concentrations of potentially respirable particles produced during routine application of talcum powder were determined. Tests were conducted both on adults exposed to talc over the whole body area and infants exposed in the napkin area. The total exposure time, the amount of powder used and the average talc concentration in the air in the region of the nares were measured. These average talc concentrations were compared with the threshold limit value (TLV) which is considered safe for industrial talc workers, and also with chronic exposure levels for experimentally exposed hamsters in which no adverse reactions were seen. The average adult exposure was 600 times less than the TLV and 500 times less than the level at which no adverse effects were seen in chronically exposed hamsters. Likewise, the average infant exposure was over 2000 times less than the TLV and over 1800 times less than the hamster no-effect level.

INTRODUCTION

When finely divided powder is dispensed from a container with a sprinkle-hole cap, airborne particles are formed in the environment of the user. Some will be 'respirable particles', but they will be at a relatively low concentration and exposure will be of short duration because of settling and dispersion. One example is the particles produced during the normal use of baby or body talcum powders.

The mass concentration of respirable particles produced during the powdering of a baby or an adult cannot be effectively measured with standard dust-sampling equipment which is used to accumulate and sample particles from more stable atmospheric areas such as mines or factories (Ayer, Sutton & Davis, 1968; Cochrane, 1972; Lippmann, 1970). The short duration and low concentration of particles produced during a single application of talcum powder poses problems and one purpose of this study was to design both the method and equipment needed to overcome these problems.

A 'respirable particle' has the potential to reach and be deposited in the lung. The probability of this occurring depends upon the aerodynamic equivalent diameter (AED) of the particle. The AED of a particle is defined as the diameter of a unit density sphere having the same aerodynamic qualities as the particle in question. In this study, a respirable particle was defined as having an AED of 10 μm or less (Ettinger, Partridge & Royer, 1970; Seltzer, Bernaski & Lynch, 1971).

A second objective of this study was to compare the average talc exposure of infants and adults to two quite different published acceptable levels of talc inhalation. Firstly, the data was compared to 3 mg/m^3, the permitted threshold limit value—time weighted average (TLV—TWA) as listed by the American Conference of Governmental Industrial Hygienists (1977). Secondly, the average talc exposures were compared to the respirable talc concentration of 8 mg/m^3 reported by Wehner *et al.* (Wehner, Zwicker & Cannon, 1977a) in an inhalation study, at which no adverse reactions were seen in chronically exposed hamsters. The TLV—TWA cited above is the weighted average concentration for a normal 8-hr working day in a 40-hr working week, to which industrial workers may be exposed safely.

EXPERIMENTAL

Equipment. Two pieces of specialized equipment were required to measure the respirable particle concentration. A prefilter to simulate the human respiratory tract by dividing the talc into respirable and non-respirable particle fractions was needed. A mass-monitoring device to remove the respirable particles from the air stream and weigh them in a very short time interval was also essential.

A 10-mm nylon cyclone was used to simulate the particle fractionation system of the human respiratory system (Caplan, Doemeny & Sorenson, 1977; Seltzer *et al.* 1971). The percentage of particles that can penetrate into the lung increases with decreasing aerodynamic equivalent diameter until other physical factors take effect and the percentage penetration reaches a plateau.

The American Conference of Governmental Industrial Hygienists and the AEC Los Alamos Scientific Laboratory have established a standard respirable dust size fractionation curve (Ettinger *et al.* 1970; Seltzer *et al.* 1971). Investigators have shown that the 10 mm nylon cyclone operating at 1·7 litres/min flow rate, has a collection efficiency for respirable particles which matches this standard (Caplan *et al.* 1977; Seltzer *et al.* 1971).

The nature of this study required mass measurement of respirable powder in quantities as low as 0·1 μg in a relatively short time span sometimes as low as 2 sec. The Thermo Systems, Inc. (TSI; St Paul, MN) quartz-crystal mass monitor Model 3210A, designed for general particle monitoring appli-

cations, was chosen for this purpose. This modular unit contains a quartz-crystal sensor nonpulsing vacuum pump to provide airflow to the 10 mm cyclone and a strip-chart recorder.

The cumulative mass of respirable particles which were fractionated by the cyclone was collected on the quartz crystal and recorded every 2 sec. Total accumulations as low as 0·1 μg could be read directly from the strip-chart recording. Measurements were made by sampling the air immediately adjacent to the nasal openings during powdering using the 10 mm nylon cyclone.

Before initiation of the study, the collection efficiency of the TSI Mass Monitor for talc particles of a respirable size was tested using the following procedure. A low density talc airborne concentration (less than 5 mg/m^3 respirable particle content) was generated in a closed chamber and sampled with the 10 mm nylon cyclone prefilter at the preferred flow rate of 1.7 litres/min. The air stream containing the respirable talc particles was then divided, with a precision splitter, and 1 litre/min passing to the mass recorder and 0·7 litres/min passing to a 13 mm membrane filter of 0·8 μm pore size. The mass recording readout on the instrument was then compared with the mass collected on the membrane filter and weighed on an electromicrobalance.

It was determined that good agreement (within 2%) could be obtained between recorder and gravimetric readings if the mass loading on the quartz-crystal sensor was kept below 15 μg talc. As the crystal mass loading increased beyond 15 μg, the talc particle deposition efficiency fell off and disparity between recorder reading and gravimetric readings increased. In this study, mass accumulations were below 15 μg for all individual tests.

Baby-powdering procedure. A group of forty-eight mothers with infants under 16 months of age, who regularly used a leading brand of talcum powder* at nappy changing time, participated in this phase of the study. Mothers were supplied with a 36 in high changing table and mat, an appropriately sized disposable napkin, and powder in its commercial 14 oz twist-top canister. All six canisters used were from the same lot.

Each mother was instructed to remove the napkin on her own infant, powder the napkin area as she normally would, and place a new napkin on the baby. When the task was completed, she was instructed to pick up her child signalling an end to the nappy-changing procedure. This procedure was repeated three times in succession for each mother–infant pair, the results being averaged. Determinations of total exposure time, amount of powder used, and average talc concentration in the air at the level of the infant's nares were made. Before running each test, the area was vacuumed to remove airborne particles left from the previous test.

To sample the airborne powder, the cyclone inlet was held next to the baby's head approximately 4 in above the surface of the changing mat (Fig. 1). Total exposure time was defined from the first shake of powder to the end of nappy changing. The investigations were carried out over two four-day time periods.

Adult powdering procedure. Twenty-three adult males and twenty-one adult females who routinely used the same leading talcum powder daily were tested in this phase of the investigation. All normally applied the powder directly to their bodies or to their hands before application to the body. Subjects were asked to shower, towel dry, enter a small (3·75 × 6·5 × 7 ft) anteroom, and apply powder from a new 14 oz twist-top canister to their bodies in their normal manner. This anteroom procedure assured a constant environment for the equipment, which is sensitive to high levels of humidity and to the temperature and humidity fluctuations that could occur with the opening and closing of the bathroom door. On entering the anteroom, each subject donned a headband with the attached cyclone which was positioned at the level of the individual's nose (Fig. 2).

Using a stopwatch, each subject was timed for the duration of the powdering procedure from the first shake of the canister to the moment body powdering was completed and the subject left the powdering anteroom. Again, the total exposure time, quantity of powder used and average talc concentration in the air at the level of the adults' nares were determined. The investigations were carried out over two four-day time periods.

*Johnson's Baby Powder®.

RESULTS

The results of the trials are summarized in Table 1. Frequency distributions of exposure concentrations for both infant and adult data are given in Tables 2 and 3. For infant exposures the time-weighted average (±SD) was 0·095 ± 0·039 mg.min/m^3 and for adult exposure, the time-weighted average was 1·727 mg.min/m^3.

The exposure factor is defined as the number of times by which the TLV—TWA for talc workers

Table 1. *Exposure of infants and adults to respirable talc particles*

	No. of subjects	Weight of talc used (g)	Exposure time (min)	Concentration* (mg/m^3)
Infants†	48	0·88 ± 0·63	0·52 ± 0·17	0·19 ± 0·084
Adults	44	8·84 ± 8·32	1·23 ± 0·55	2·03 ± 1·49

*Average concentration, in mg/m^3, was calculated by dividing the weight of respirable particles collected by the volume of air sampled for each subject, and averaging the results. The flow rate to the mass monitor was 1 litre/min; therefore, the exposure time in minutes was equal to the air volume in litres.

†Because of the very low airborne respirable concentrations during the infant powdering, it was necessary to use the mean of three consecutive tests on each infant.

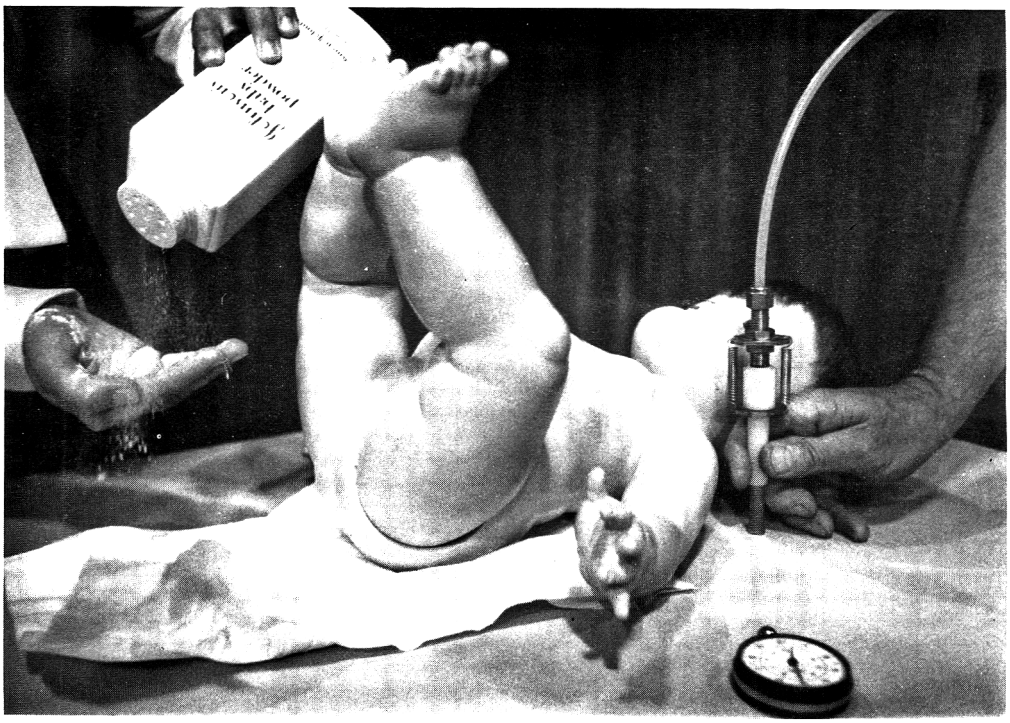

Fig. 1. Simulation of particle collecting cyclone positioned at the level of baby's nares during nappy-change powdering.

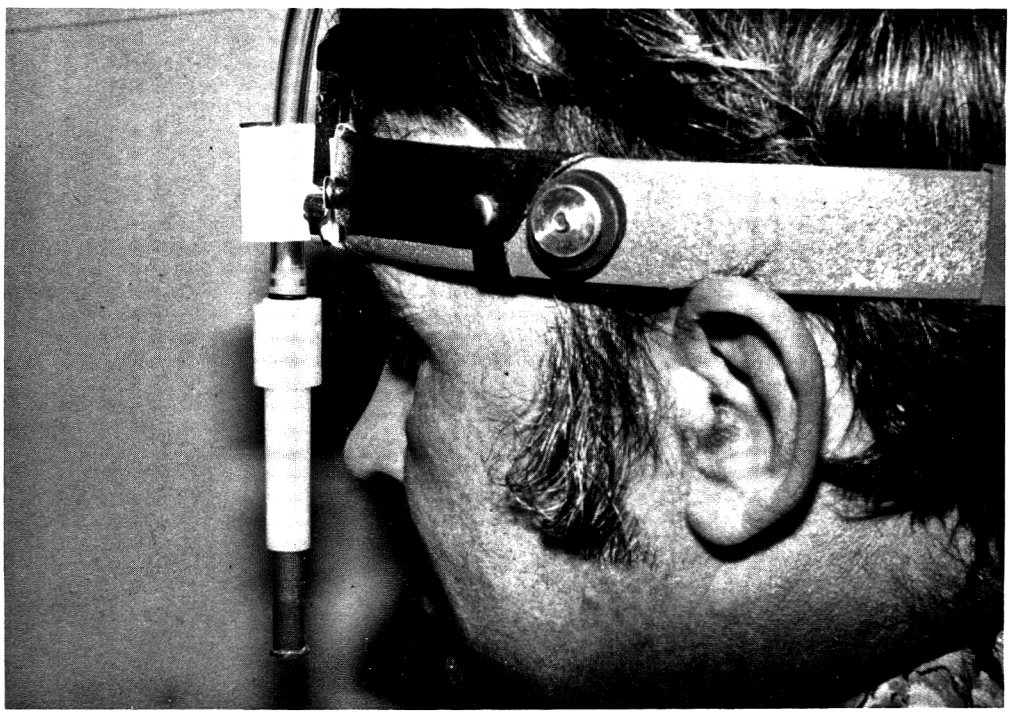

Fig. 2. Particle collecting cyclone attached to a headband and positioned at the level of the subject's nares during adult whole-body powdering.

Table 2. *Frequency distribution of infant exposure (mg.min/m³) to respirable talc particles*

Exposure levels*† (mg.min/m³)	No. of infants within exposure level	Percentage of infants within exposure level
0·10–0·19	4	8·33
0·20–0·29	15	31·25
0·30–0·39	19	39·58
0·40–0·49	6	12·50
0·50–0·59	3	6·25
0·60–0·69	0	0·00
0·70–0·79	1	2·08

*Data for the mean of three exposures.
†The time-weighted average (±SD) for three exposures was 0·285 ± 0·117 mg.min/m³; the average for one exposure was 0·095 ± 0·039 mg.min/m³. Because these results approximated a normal distribution, parametric statistical procedures were applied to the raw data.

Table 3. *Frequency distribution of adult exposure (mg.min/m³) to respirable talc particles*

$\log_e$* exposure levels (mg.min/m³)	No. of adults within exposure level	Percentage of adults within exposure level
−1·80 to −1·36	1	2·27
−1·35 to −0·91	3	6·83
−0·90 to −0·46	4	9·09
−0·45 to −0·01	3	6·82
0·00–0·44	9	20·45
0·45–0·89	9	20·45
0·90–1·34	6	13·64
1·35–1·79	5	11·36
1·80–2·24	2	4·55
2·25–2·69	2	4·55

*The adult exposure data closely approximated a log-normal distribution. Therefore, a $\log_e$ transformation was made to "normalize" the data and parametric statistical procedures were applied to the transformed data. The $\log_e$ of the time-weighted average exposure (±SD) was 0·5464 ± 0·9339 and antilogs allowed estimation of the original population, the time-weighted average being 1·727 mg.min/m³.

exceeds the time-weighted average concentrations determined for infants and adults in this study. Exposure factors were calculated by dividing the weekly TLV concentration for talc workers by the calculated weekly concentrations for either infants or adults. The time-weighted exposures for infants and adults were converted to weekly exposure concentrations by multiplying by the number of applications per day and by seven (days/week) and dividing by 60 (min/hr). J. N. Sivertson (unpublished data, 1976) has shown that mothers, who are regular users of baby powder, powder their infants (1 to 24 months old) an average of five times/day. One powder application each day was assumed for adults. The average values obtained were 0·055 and 0·20 mg.hr/m³ for infants and adults respectively. The corresponding 90% tolerance limits for the 95th percentiles were found to be 0·099 and 1·23 (Diem, 1962). The talc TLV of 3 mg/m³ was converted to a weekly exposure for the talc workers assuming a 40-hr working week. This figure of 120 mg.hr/m³/wk was then divided by the average infant or adult weekly time-weighted values, and the exposure factors were calculated to be 2182 for infants and 600 for adults. Exposure factors calculated from the 90% tolerance limit for the 95th percentile for each group were 1212 and 98 respectively.

The above exposure data were also compared with the data of Wehner (Wehner *et al.* 1977a) from a hamster inhalation study. In this case the no-effect level for the hamsters 100 mg.hr/m³/wk exceeded the exposure levels of the infants and adults 1818 and 500 times respectively.

DISCUSSION

The background level of respirable particles in the area of the tests was monitored and found to be less than 0·003 mg/m³. It was determined that background particles would increase the concentration by a maximum of 4% under the most severe conditions encountered, e.g., 1·5 min of exposure and 0·1 μg total respirable mass collected. Therefore, background was not considered in calculating the exposure concentrations.

It was interesting to observe the different methods of powder application; three-quarters of the mothers applied the powder directly to the baby's body and the remainder placed the powder in their hands and then on the baby. One mother applied the powder to the napkin. Analysis of data resulting from the various application methods showed no significant differences in any of the monitored parameters.

The average adult exposure concentration (2·03 mg/m³) was over ten times greater than that found for infants (0·19 mg/m³). Adults were also exposed for a longer time period, 1·23 min compared with 0·52 min for the infants. On average, men tended to powder in a shorter time than women, but to use more powder and create greater airborne respirable particle concentrations.

It is noteworthy that Wehner *et al.* (1977a) were unable to produce any talc-related toxicity in hamsters with extensive exposures to 8 mg/m³/wk of respirable talc. The same group later demonstrated that although the talc deposited in the deep lung, it was cleared with a biological half-life of 7–10 days (Wehner, Wilkerson, Cannon, Buschbom & Tanner, 1977b).

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URINARY SILICON EXCRETION BY RATS FOLLOWING ORAL ADMINISTRATION OF SILICON COMPOUNDS

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Abstract—The rate and extent of urinary excretion of silicon was determined in rats after oral administration of magnesium trisilicate, food-grade sodium aluminosilicate, sodium silicate or Zeolite type A. The materials were given in doses of 0, 40, 200 or 1000 mg/kg body weight. Urinary silicon excretion increased rapidly after dosing and peak excretion rates occurred within 24 hr in all test groups. Zeolite A had the most rapid urinary excretion rate (half-life, 6–8 hr) followed by magnesium trisilicate (half-life, 16–20 hr), sodium silicate (half-life, 24 hr) and sodium aluminosilicate (half-life, 38 hr). First-order excretion kinetics were followed for all four materials. When expressed as a percentage of dose the total urinary silicon excreted was roughly equal for magnesium trisilicate, sodium silicate and Zeolite A but considerably less for sodium aluminosilicate. Urine of rats dosed with sodium aluminosilicate or Zeolite A did not show any detectable increase in aluminium, indicating that the silicon that was excreted was a breakdown product of the parent molecule. Since it is known that aluminium when administered either ip or iv is excreted in urine, this is evidence for decomposition of the test material in the gastro-intestinal tract. For all four test materials urinary silicon excretion increased with dose level. However, the magnitude of this increase (two- to eightfold) was not as great as the increase in the amount dosed (25-fold) so that the percentage of silicon excreted decreased as the dose was increased. This suggested that some process in the absorption or excretion of silicon was becoming saturated. These results are discussed with respect to the comparative safety of these four silicates.

INTRODUCTION

Silicon is found in all natural waters (Krauskopf, 1967) and in almost all terrestrial and aquatic organisms (Allison, 1968). Therefore, man cannot avoid silicon exposure. Recent studies with chickens (Carlisle, 1972) and rats (Schwarz & Milne, 1972) show that silicon is essential for normal growth. Silicon may also protect against atherosclerosis in man (Schwarz, 1977). As with many other essential elements, certain chemical forms of silicon may be toxic if ingested or inhaled in large amounts (King & Belt, 1938), although the chronic oral ingestion of small amounts of many siliceous materials is generally considered safe as evidenced by the number of silicates on the FDA GRAS list (Code of Federal Regulations, Title 21, Part 170). Few comparative studies of different silicon compounds have been recorded in the literature. Keeler & Lovelace (1959), using rats, and Sauer, Laughland & Davidson (1959), using guinea-pigs, measured urinary silicon after the oral and ip administration of several silicon-containing compounds. The excretion rates were not precisely determined, nor were detailed dose-response data obtained.

Our interest in silicates (specifically, Zeolite type A (ZA)) stems from work on the development of replacements for phosphates in laundry detergents (Savitsky, 1977; Schwuger & Smolka, 1976). One approach to the safety assessment of a new ingredient is to compare it with similar, more familiar materials, for which safety is generally recognized. Fortunately, there are several silicon compounds which qualify for this comparison with Zeolite A. One of these is the GRAS substance sodium aluminosilicate (SAS), for which the estimated average daily consumption is 1·3 g (Tracor-Jitco, 1973). Another such compound, magnesium trisilicate (MgTS) is an over-the-counter antacid which has been used by man for more than 40 yr (Mutch, 1936). A third material that can be included, sodium silicate (SS), is a common ingredient in granular laundry detergents and is widely used in a variety of industrial processes (*Merck Index*, 1968).

Since the hazards of ingested silicates are generally limited to effects on the kidney and bladder (Emerick, Kugel & Wallace, 1963; Tracor-Jitco, 1973), and since the urine is the major excretion route for silicon, we studied the urinary excretion of silicon after the ingestion of silicon-containing substances, as a means of assessing the exposure of these target organs. For SAS and ZA, aluminium was also determined to provide a means of detecting changes in the structural integrity of these two materials.

EXPERIMENTAL

Materials. The sodium aluminosilicate (SAS) used in this study was obtained from J. M. Huber Corporation (Havre de Grace, MD) under the tradename of Zeolex®. Huber Corporation specified that this compound met all the purity and quality specifications of the *Food Chemicals Codex*; our analyses showed that it contained 30·0% silicon and 5·4% aluminium. The sodium silicate (SS) was manufactured by Philadelphia Quartz Company (Valley Forge, PA) under the tradename of Britesil® C24; our analyses

showed that it contained 25·9% silicon. Magnesium trisilicate (MgTS) was USP, (Lot CLJ), from Mallinckrodt Chemical Company (St. Louis, MO); it contained 20·2% silicon by analysis. The Zeolite A (ZA) is a sodium aluminosilicate manufactured by J. M. Huber Corporation under the name of Arogen 2000. Its identity was confirmed by infrared spectroscopy. It contained 14·7% silicon and 16·4% aluminium by analysis. This material also contained a minor amount ($\leq 5\%$) of hydroxysodalite but no measurable levels of other zeolite materials, (e.g. types X, Y or PLB). The empirical formula of ZA is $Na_{12}(AlO_2)_{12}(SiO_2)_{12}.27H_2O$.

Animals and diets. Male Sprague-Dawley Cox rats weighing 240–260 g obtained from Laboratory Animal Supply Co., Indianapolis, IN, were acclimatized for 4–5 days in groups of three and were provided with Purina Rat Chow (*c.* 1500–2000 ppm silicon) and tap water (*c.* 5 ppm silicon) *ad lib.* During the next 7 days the rats were gradually introduced to a liquid diet, so that by the end of this period they were receiving no solid food. The composition of the diet was as follows: sucrose (25·4%), non-fat dry milk (18%), water-soluble vitamins (no. 18; 0·6%) (Mattson & Nolen, 1972), Salt Mix, USP XIV (3%), soya-bean oil (20%), and water (33%). The liquid diets were necessary to prevent contamination of urine samples by feed during the collection period, and assured a consistent level of dietary silicon intake among the test and control animals both before and during sample collection. Previous studies have not used this approach to reduce variation between animals (Keeler & Lovelace, 1959; Sauer *et al.* 1959).

For the study of particulates in urine, four male Charles River rats (from Charles River Labs, Wilmington, MA) weighing 240–260 g were used. Acclimatization was as described above.

Dosing and urine collection procedures. Rats were fasted for 17–18 hr, then dosed using a no. 10 French rubber feeding tube attached to a 3-ml plastic syringe. Concentrations (w/w) of dosing materials were adjusted so that all groups received the same dosage volume (10 ml/kg). The actual weights administered were calculated by difference. All suspensions were prepared in quartz-distilled (QD) water which contained <0·5 ppm silicon and aluminium. Rats were dosed with 40, 200 or 1000 mg of test material/kg body weight. For each study a group of four or six control rats were given 10 ml QD water/kg body weight. Tail cups to collect faeces were used to prevent contamination of urine samples. Rats were placed in stainless-steel metabolism cages for urine collections. Urine was collected in 60-ml plastic vials that had been cleaned with 6 N-HNO_3 (6-hr soak), 0·1% EDTA (single rinse), QD water (three rinses) and acetone (one rinse). Individual cages were rinsed with 5–10 ml QD water after each collection and the rinses were combined with the urine. In the study of silicon particulates, rats were anaesthesized with CO_2 after the 8-hr collection period and the abdominal cavity was opened. Urine remaining in the bladder was aspirated using a syringe (No. 18 g needle), and added to the collection vial. Rats were then exsanguinated from the aorta.

Filtrations. Urine from rats in the particulate study was brought to 37°C in a water bath before filtration. A 3-ml aliquot was filtered using a 0·4-μm Nuclepore filter and a Micropore filtration apparatus. Residual urine was washed through the filter using methanol (3 ml). Filters and an unfiltered urine sample were then assayed for silicon as described below.

Analytical method. For the determination of silicon and aluminium in urine, the samples were dry-ashed, fluxed with Na_2CO_3, and then dissolved in hydrochloric acid. The concentrations of silicon and aluminium in solution were measured by induction-coupled RF plasma optical emission spectrometry. The 251·6 nm and 394·4 nm emission lines were used for silicon and aluminium, respectively. This technique was chosen because it can determine silicon and aluminium simultaneously, it is subject to minimal interferences, it is sensitive, and it is compatible with the acid solutions obtained from the sample preparation step. Typical detection limits were 0·2 ppm for both silicon and aluminium. The method was developed by F. Lichte, T. W. Osborn and S. Hopper (unpublished data 1978).

Statistical analysis. Group means were compared by Student's *t*-test, except that when the values were not normally distributed a non-parametric test was used (Gibbons, 1971). The rates of excretion were fitted to zero-order and first-order kinetic equations (Barr, Goodnight, Sall, & Hellwig, 1976).

RESULTS AND DISCUSSION

Urinary excretion of silicon

Figure 1 shows that the excretion of silicon in urine markedly increased after single oral doses of any of the four test substances. The control values, shown at the bottom of the figure, have been subtracted from each test value shown. A separate control group was used for each study. Silicon excretion was most rapid during the first 24 hr after dosing (collection period 1). The test material which gave the greatest increase in silicon excretion within a particular collection period varied depending on the dose level. Further, the rate at which the urinary silicon excretion returned to control levels also varied between compounds. For example, for the first collection period (0–24 hr) at a dose level of 40 mg/kg the order of silicon excretion was SS $\geq$ MgTS $\geq$ ZA $\gg$ SAS; at 200 mg/kg the order was ZA > MgTS > SAS (SS not tested at this dose), and at 1000 mg/kg it was ZA > SS > MgTS > SAS. This pattern changed substantially for the 24–48-hr collection period. Here, at 40 mg/kg. MgTS > SS > SAS = ZA; at 200 mg/kg, SAS = ZA > MgTS; and at 1000 mg/kg, SS > SAS > MgTS > ZA. A similar pattern to that of the 24–48-hr period was seen again for the 48–72-hr collection.

The percentage of the dose excreted must be considered in the context of excretion rate to predict the concentration of silicon occurring in urine at a particular time after dosing. The excess urinary excretion of silicon, expressed as a percentage of the dose, is also shown in Fig. 1 (these values are shown in parentheses). Rats dosed with MgTS, SS, SAS, and ZA all excreted urinary silicon in excess of background levels. For MgTS, SS and ZA, while the amount of excess urinary silicon increased with increasing dose, the percentage of the dose excreted decreased with increasing dose. The pattern observed for SAS was

different, the percentage of silicon that appeared in urine varied independently of the dose. This may have been an artefact resulting from a slightly higher urinary silicon excretion by the control animals in the SAS study. For example, if the level of urinary silicon excreted during the collection period by the controls in this study was 384 μg rather than 621 μg (384 μg is the average 72-hr control value for the other three compounds), the urinary silicon excretion at 40, 200 and 1000 mg SAS/kg would have been 12·4, 5·2 and 1·3%, respectively. This is the pattern followed by ZA, MgTS and SS. The fact that the increase in urinary excretion of all four materials was not in direct proportion to the increase in dose may have been due to the saturation of some process, related either to the absorption or to the excretion of silicon. Similar findings were reported by King, Stantial & Dolan (1933) who administered silicic acid to dogs and found that increasing the dose caused a smaller fraction of the silicon to be excreted in urine. Comparable data in humans are available only for MgTS. Page, Heffner & Frey (1941) administered MgTS to volunteers at a dose level of 35 mg/kg/day for 4 days and then continued to collect urine for 48 hr after the last dose. The total excess silicon recovered in urine averaged 5·2%. Although these data are not directly comparable with our single-dose data, it seems that man absorbs less silicon from MgTS than does the rat.

The changing pattern of silicon excretion with time in the groups of rats given the four silicon materials, indicates that the excretion rates were different among these groups. The urinary silicon excretion half-lives, determined by the method of Barr *et al.* (1976), were: SAS, 38 hr; SS, 24 hr; MgTS, 16–20 hr; and ZA, 6–8 hr. The dose-response data that were obtained for MgTS and ZA (only) indicated that urinary excretion

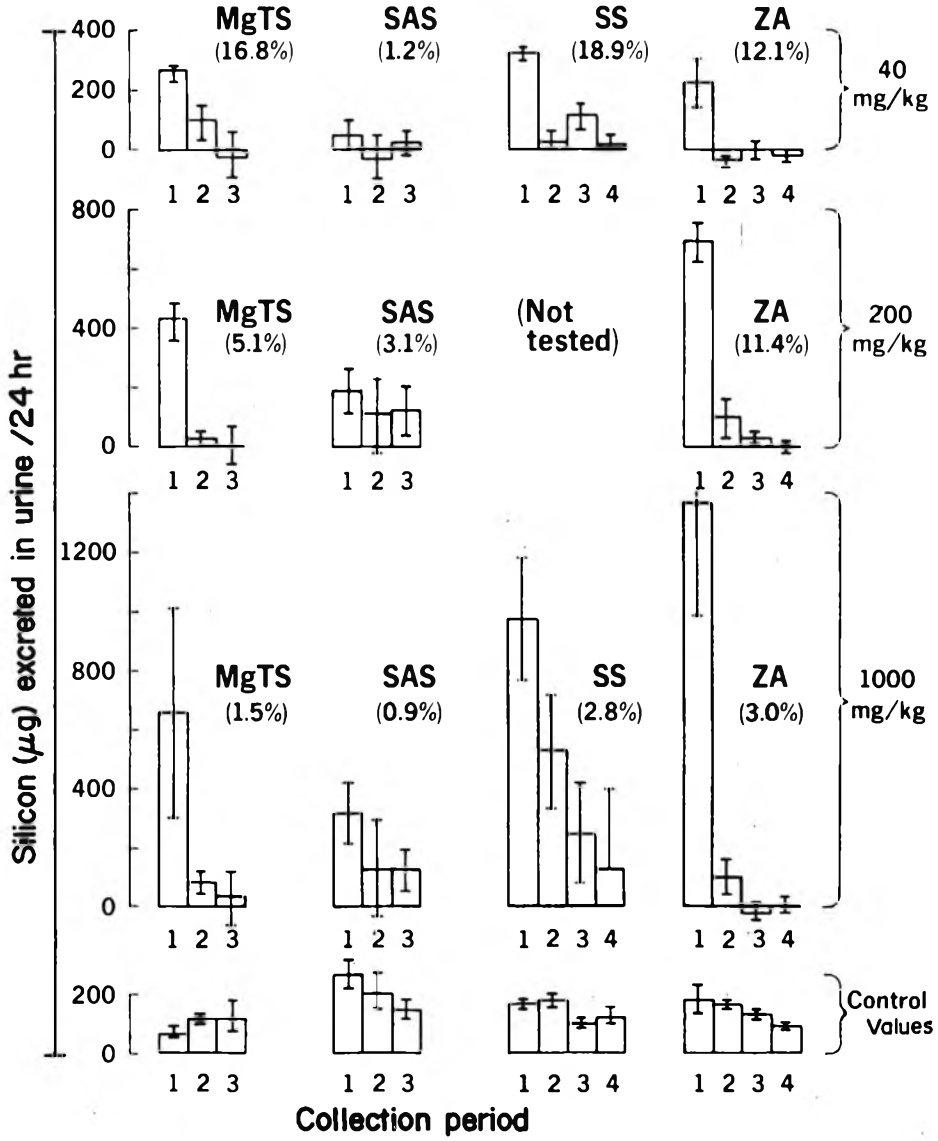

Fig. 1. Urinary excretion of silicon by rats after oral dosing of magnesium trisilicate (MgTS), sodium aluminosilicate (SAS), sodium silicate (SS) or Zeolite A (ZA). The control values have been subtracted from each test value shown. The numbers in brackets are the percentages of the silicon dose recovered in urine (control values subtracted). Collection periods 1, 2, 3 and 4 refer to 0–24, 24–48, 48–72, and 72–96 hr after dosing, respectively. Bars on control values represent ± one standard error (N = 4).

half-lives were independent of dose within the 40 to 1000 mg/kg dosage range, and followed first-order excretion kinetics. This lack of dose-response in urinary excretion half-life indicates that the limiting or saturable factor, suggested by the percentage excretion data in Fig. 1, is not the kidney elimination step since if this were the case the smaller doses would be excreted more rapidly. Another possible saturable factor is the rate of gastro-intestinal absorption. However, one could not then explain the large differences in half-life between the four substances. One other possibility is the rate of production of soluble or absorbable silicon in the gastro-intestinal tract. The four materials probably undergo acid hydrolysis and produce soluble (absorbable) forms of silicon at different rates characteristic for each compound. In separate studies we have determined that the acid hydrolysis of ZA is much faster than the hydrolysis of SAS. At higher doses the same rate of hydrolysis occurs, but there is much more material available, so that a large quantity is absorbed.

Exposure of the urinary tract during silicon excretion is a function of both concentration and length of exposure. Assuming a similar level of total absorption, one would expect that the highest urinary concentration would occur in rats dosed with the material having the shortest half-life, i.e., ZA (also assuming other factors such as volume of distribution, binding, etc. are constant). The 0–24 hr data (Fig. 1) show that this is the case. However, the net concentration of silicon in urine at 24–48 and 48–72 hr is lower for rats dosed with ZA compared to SS and even SAS, which is absorbed to a much lower degree. SS, having a relatively high percentage absorption and relatively long half-life, produced the greatest urinary silicon concentration between 48 and 72 hr. If the acute toxic effects of silicon on the kidney are mostly related to length of exposure, SAS and SS with their longer half-lives would be predicted to have the greatest toxicity. However, if the effects are related to the peak concentration of urinary silicon, then MgTS and ZA would have the greatest toxicity. Data presented by Emerick *et al.* (1963) suggest that silicon toxicity in the urinary tract is manifested via formation of kidney and bladder calculi, and these authors showed a good correlation between polymeric silicon and the formation of calculi in rats fed tetraethylorthosilicate. Since in the present studies ZA gave the highest peak levels of silicon it was of interest to find out whether particulate, or filterable, forms of silicon were produced during the time of maximum excretion in rats dosed with ZA. These data are shown in Table 1. Note that while the total silicon concentration increased sharply with dose, the particulate silicon was not increased above control levels. We would, therefore, predict that toxic effects in the urinary tract would not result from single high doses of Zeolite A.

Table 1. *Particulate and total silicon in rat urine after the administration of Zeolite A*

Dose (mg/kg)	Silicon (μg/ml urine)	
	Total	Particulate
Control	6·5 ± 0·6	2·2 ± 1·1
40	26·2 ± 3·4	1·4 ± 0·3
200	64·7 ± 6·8	1·6 ± 1·3
1000	80·9 ± 13·5	2·2 ± 0·8

Values are means ± SEM for four rats. The urine was collected during the 8-hr immediately after dosing.

Urinary excretion of aluminium

For SAS and ZA the analysis of urine also included aluminium since absorbed aluminium, like silicon, is excreted mostly by the kidney (Myers & Morrison, 1928). Ondreicka, Kortus & Ginter (1971) suggested that, because of the poor absorption of aluminium, very high levels of aluminium would have to be administered to test animals for any increase in urinary aluminium to be detected.

In our studies we could not detect any significant increases in urinary aluminium in rats administered SAS or ZA at levels of 40–1000 mg/kg. Daily urinary aluminium excretion averaged 17·7 ± 3·2 μg (±SEM) for control rats, 15·1 ± 4·3 μg for SAS-treated rats, and 12·3 ± 1·1 μg for ZA-treated rats during the 72-hr period following dosing. The aluminium detection limits would have permitted the detection of 0·03 to 0·8% of the dose following doses of 40–1000 mg/kg of SAS or 0·01 to 0·2% of dose following 40–1000 mg/kg ZA. By inference, the excreted species of silicon, then, cannot be the parent compound (in the case of ZA or SAS). Since it has been shown in ip- and iv-administered aluminium is excreted in the urine (Myers & Morrison, 1928) it is most likely that breakdown of SAS and ZA occurred in the gastro-intestinal tract, and only the silicon portion was absorbed.

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STUDIES ON THE ACTION OF HISTAMINE RELEASE BY PERSULPHATES

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Abstract—Potassium dipersulphate, ammonium dipersulphate and potassium monopersulphate release histamine from rat peritoneal mast cells *in vitro*, and from guinea-pig skin *in vitro* and *in vivo*. Potassium dipersulphate releases histamine from rat peritoneal mast cells by a slow, dose-dependent, non-cytolytic mechanism. By contrast, potassium monopersulphate releases histamine by a rapid cytolytic mechanism. Ammonium dipersulphate-induced histamine release appears to have similarities with histamine release induced by both potassium salts. All three salts are considerably less potent histamine releasers than compound 48/80. Results from the experiments with guinea-pig skin *in vitro* support a selective mechanism for histamine release by potassium dipersulphate.

INTRODUCTION

Allergic contact dermatitis, chronic and generalized urticaria, and early and late onset asthmatic reactions have been reported in some individuals (bakers or hairdressers) following occupational exposure to 'persulphates' or the use of hair-bleach preparations containing these salts (Bonnevie, 1939; Brubacker, 1972; Calnan & Shuster, 1963; Fisher & Dooms-Goossens, 1976; Forck, 1968; Pepys, Hutchcroft & Breslin, 1976; Schulz, 1967; Sidi, Gervais, Bourgeois-Spinasse & Gervais, 1966). Patch tests with the relevant salts have been used to confirm the allergic contact dermatitis, but immediate urticarial reactions preceding the typical delayed reaction have been observed in a number of cases. However, the nature of this early reaction, also observed on scratch and intracutaneous testing, and the asthmatic reactions to these salts, have remained uncertain.

Calnan & Shuster (1963) suggested that 'persulphate' is a weak histamine liberator, although the action of ammonium dipersulphate on isolated skin slices (Mahzoon, Yamamoto & Greaves, 1977) was subsequently thought to occur by a non-specific toxic action on mast cells. The present report describes the results of experiments to investigate histamine release by mono- and dipersulphates from isolated rat peritoneal mast cells, and from guinea-pig skin *in vivo* and *in vitro*. The results suggest that the dipersulphate ion releases histamine by a non-cytolytic mechanism, which resembles in some respects the histamine release evoked by compound 48/80.

EXPERIMENTAL

Materials. Potassium dipersulphate ($K_2S_2O_8$) was obtained from BDH Chemicals Ltd., Poole, Dorset, ammonium dipersulphate $(NH_4)_2S_2O_8$ from Hopkin and Williams Ltd., Romford, Essex, compound 48/80 from Sigma Chemical Co., St. Louis, MO, disodium cromoglycate (DSCG) from Fisons Ltd., London, and mepyramine maleate from May and Baker Ltd., Dagenham, Essex. Potassium monopersulphate ($KHSO_5$) was prepared by Dr. A. H. Clements, Unilever Research Laboratory, Port Sunlight.

Isolation of rat peritoneal mast-cell preparations and measurement of histamine release by persulphates. Colworth Wistar-derived rats (approximately 200 g body weight) were used for the isolation of the peritoneal mast-cell preparations. Mast cells were isolated essentially as described by Perelmutter & Khera (1970). Rats were killed by carbon dioxide asphyxiation and their peritoneal cavities were irrigated with 25–30 ml of either Eagles minimum essential medium (MEM), pH 6·8 (Eagle, 1959), or a buffered salt solution (BSS) of the following (mM) composition: NaCl 139, KCl 2·4, $CaCl_2$ 0·8, Sørensen phosphate buffer 6·7 (pH 6·8) and dextrose 5·6. Each medium contained ethylenediaminetetraacetic acid, disodium salt (EDTA) at a level of 1·3 mM. All reagents were of AnalaR quality. The peritoneal washings from at least five rats were pooled, centrifuged (250 g) at room temperature for 10 min and resuspended in a suitable volume of Eagles MEM or BSS without EDTA.

The mast cells, used without further purification, were identified by staining with crystal violet and counted in a Neubauer chamber. It was estimated that mast cells comprised 5–10% of the total peritoneal-cell preparation. The volume of the cell suspension was adjusted to give a concentration of 1×10^5 mast cells/ml.

Aliquots (100 μl) of test substance dissolved in the appropriate medium at various concentrations were incubated with 0·5-ml aliquots of the mast-cell suspension under a variety of conditions (of temperature, pH and time). Following incubation, the cell suspensions were cooled rapidly in an ice-bath or by the addition of ice-cold medium, and each sample was centrifuged at 700 g for 5 min in the cold. Aliquots (0·5 ml) of the supernatant were added to an equal volume of 0·8 M-perchloric acid and assayed for histamine by an automated spectrofluorometric assay based on that reported by Evans, Lewis & Thomson

(1973). In any procedure, three or more replicates were tested for each treatment and all experiments were repeated at least twice using different cell pools.

The release of histamine was expressed as a percentage of the total histamine content of the mast-cell aliquot, determined by addition of an equal volume of 0·8 M-perchloric acid. The results were corrected for spontaneous histamine release in medium alone (normally between 5 and 8%) and for any interference with the histamine assay due to the test substance or vehicle. The stability of histamine in the presence of the test material was also examined.

In additional experiments, cell pellets were treated and then washed twice in Eagles MEM and stained with neutral red. Cells were examined for evidence of degranulation and/or disruption of the cell membrane.

Histamine release elicited by the test substance(s) was compared with that evoked by compound 48/80 under the same conditions. All test materials were prepared in the appropriate medium immediately before use.

Haemolytic action on sheep erythrocytes. Sheep erythrocytes (0·5-ml aliquots at a count of 5×10^8/ml) in 0·9% saline were incubated at 37°C for 30 min with 100-μl aliquots of the test substances in 0·9% saline to give a final concentration of 0·33–2·7 mg/ml. After incubation the erythrocyte suspensions were centrifuged at 1000 **g** for 5 min and examined for haemolysis.

Histamine release from guinea-pig skin in vitro. Tests for histamine release from sliced guinea-pig skin *in vitro* were essentially as described by Yeoh, Tay & Greaves (1972). Abdominal skin slices, 500 μm thick, were prepared with a Castroviejo Electro-Keratome. Duplicate skin slices were incubated in 0·5 ml of Tyrode solution, containing (in mM concentrations) NaCl 137, KCl 2·7, $CaCl_2$ 1·8, $MgCl_2$ 1·0, NaH_2PO_4 0·4, $NaHCO_3$ 12·0 and dextrose 5·6 and having a pH of 7·4, with a range of concentrations of the test substances (0·2–16 mg/ml) or with compound 48/80 (0·2 mg/ml) for 30 min at 37 or 4°C. The reaction was stopped by cooling, removing the supernatant and adding it immediately to an equal volume of 0·8 M-perchloric acid. The amount of histamine released, corrected for spontaneous histamine release and any assay interference, was expressed as a percentage of the total histamine per sample. The total histamine was calculated from the sum of released and residual histamine (the latter determined by heating the skin slices in 0·4 M-perchloric acid for 10 min followed by cooling for 20 min).

Effect of persulphates on guinea-pig skin in vivo. Aliquots (0·05 ml) of the test substances (in concentrations of 4, 8 and 16 mg/ml saline), compound 48/80 (80 μg/ml), histamine base (60 μg/ml) and saline (vehicle control) were injected intradermally into the shaved dorsal skin of four Colworth guinea-pigs (350 g body weight), which had been treated iv 20 min before with Evans Blue dye (2·5% in 0·45% saline, 1·2 ml/kg). The injection sites were randomized and the animals were killed 40 min after the last injection. The dorsal skin was removed and the size and intensity of the reactions were assessed from the inner surface of the skin. The size of the lesion was quantified by measurement of two diameters (mm) and by recording the amount of extravasated dye on the arbitrary scale (faint blue, pale blue, blue, deep blue, white centre, red centre). In a repeat experiment, a further four guinea-pigs were treated iv with mepyramine maleate (8 mg/kg) in saline 45 min before the intradermal injection.

RESULTS

Characteristics of peritoneal harvest of mixed cells

The mean total histamine content from mast cells in the peritoneal harvest of mixed cells was 26·2 ± 1·1 μg histamine base/10^6 mast cells (n = 83). Histamine was released in a dose-related manner by incubation with compound 48/80 tested over the 0·13–0·83 μg/ml range, 54·95 ± 3·68% (n = 29) of the total histamine content being released by the highest concentration of 48/80 tested (0·83 μg/ml) in Eagles MEM. (Values for histamine release are expressed as the mean ± SEM).

Histamine release from rat peritoneal mast cells by mono- and dipersulphates

Histamine was released by all three persulphates (potassium mono- and dipersulphates and ammonium dipersulphate) in a dose-dependent manner from rat peritoneal mast cells suspended in Eagles MEM (Figs 1a–c). The pH of the incubation medium was unaltered by the presence of potassium and ammonium dipersulphate at the concentrations tested (0·33–2·7 mg/ml). Potassium monopersulphate at the higher concentrations tested (2·3 and 2·7 mg/ml) reduced the pH of the incubation medium to approximately pH 4. Adjustment of the pH of these solutions to neutral with 1 M-sodium hydroxide before addition to the mast-cell suspension, however, did not alter the shape of the dose-response curve. The incubation time (40 min) used in tests to obtain the standard curves was chosen on the basis of the results of the time-course experiments described below.

Examination of mast cells after treatment with the persulphates showed two types of effect. Potassium monopersulphate at acid or neutral pH caused disruption of the mast-cell membrane. Potassium dipersulphate caused degranulation of the mast cell without disruption of the cell membrane. The morphology of the mast cells treated with potassium dipersulphate resembled that of mast cells treated with compound 48/80 under the same conditions. Ammonium dipersulphate treatment caused an alteration of the granules, but did not result in any apparent degranulation or disruption of the cell membrane.

The temperature-dependence of histamine release by potassium monopersulphate and by potassium and ammonium dipersulphate is shown in Fig. 2. Histamine release by potassium dipersulphate was inhibited at 4 and 45°C. When cells were incubated at 37°C following a period of incubation at 4°C, the percentage histamine release was approximately half that normally observed at 37°C. The incubation period at 37°C in this experiment, however, was 25 min compared to the 40-min incubation time used to determine the dose-response curves. Cells incubated at 45°C with potassium dipersulphate did not regain their ability to release histamine when the incubation temperature was subsequently reduced to 37°C. The

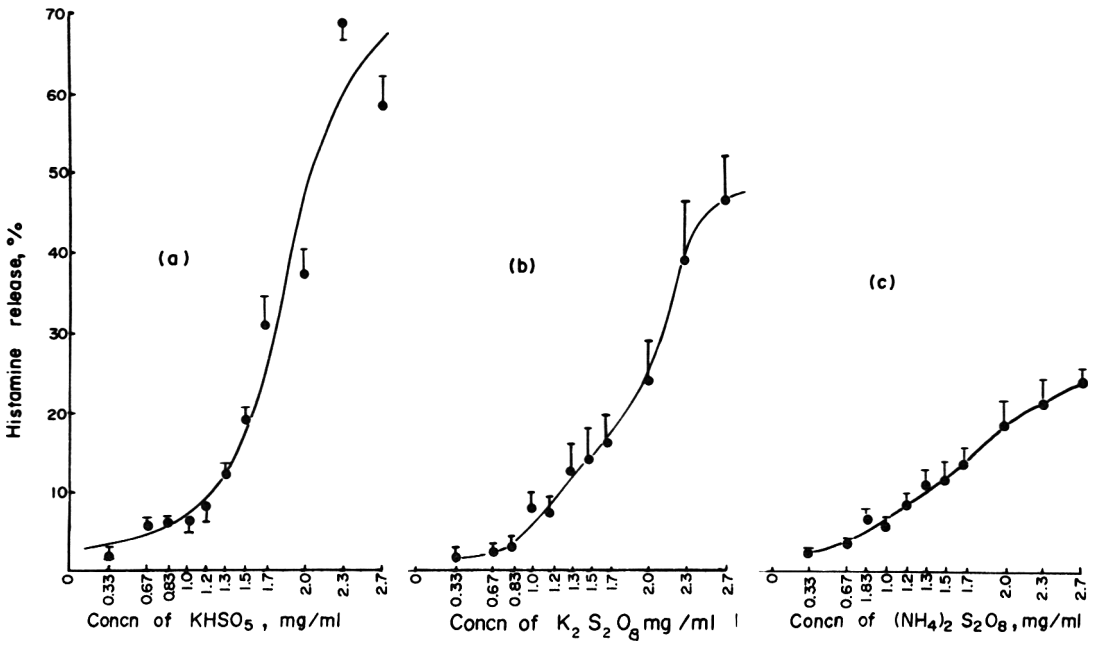

Fig. 1. Histamine release induced by increasing concentrations of (a) potassium monopersulphate, (b) potassium dipersulphate and (c) ammonium dipersulphate on rat peritoneal mast cells incubated in Eagles MEM for 40 min at 37°C. The pH of potassium monopersulphate solutions was not adjusted to neutral before addition to the mast cell aliquots. Each point represents the mean ± SEM of three to eight separate experiments with different cell pools, all values having been corrected for spontaneous histamine release in Eagles MEM.

temperature-dependence of histamine release for potassium dipersulphate was similar to that observed for compound 48/80. Potassium monopersulphate released histamine from mast cells when incubated at 4 or 45°C. The amount of histamine released was less at 4 than at 37°C, but the release was not completely inhibited. Slightly more was released at 45 than at 37°C. The temperature profile of histamine release by ammonium dipersulphate was similar to that of potassium monopersulphate.

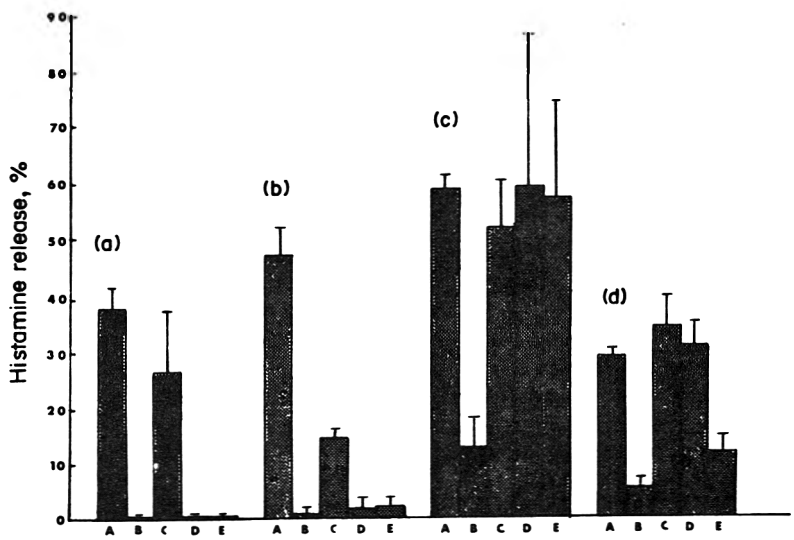

Fig. 2. Effect of temperature on histamine release from rat mast cell suspensions in Eagles MEM incubated with (a) compound 48/80 (0·33 μg/ml), (b) potassium dipersulphate (2·7 mg/ml), (c) potassium monopersulphate (2·7 mg/ml, not pH adjusted) or (d) ammonium dipersulphate (2·7 mg/ml) under the following conditions: (A) at 37°C for 40 min with releasing agents; (B) at 4°C for 40 min with releasing agents. (C) at 4°C for 15 min with releasing agents and then at 37°C for an additional 25 min. (D) at 45°C with releasing agents for 40 min and (E) at 45°C with releasing agents for 15 min and then at 37°C for an additional 25 min. Heights of histograms represent means ± SEM of histamine release (%) in two or three separate experiments with different cell pools, all values having been corrected for appropriate spontaneous histamine release in Eagles MEM.

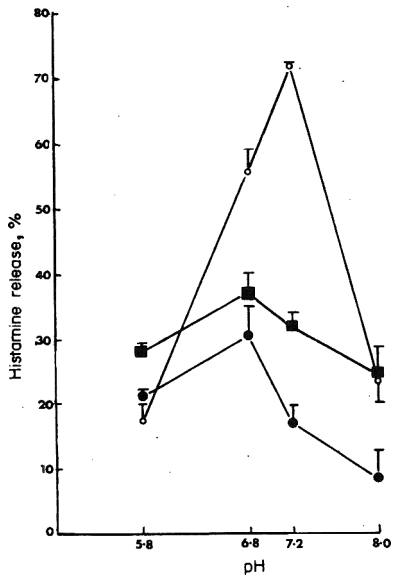

Fig. 3. Effect of pH on histamine release by rat mast-cell suspensions incubated for 30 min in BSS (Sørensen phosphate buffer) at 37°C in the presence of compound 48/80 (0·17 μg/ml; ○), potassium dipersulphate (2·7 mg/ml; ■) or ammonium dipersulphate (2·7 mg/ml; ●). Where necessary, solutions of the releasing agents were adjusted to the correct pH prior to addition to the mast-cell aliquots. Each point represents the mean ± SEM of three separate experiments with different cell pools, all values having been corrected for appropriate spontaneous histamine release in BSS at the relevant pH.

At the concentrations used, none of these persulphates interfered with the fluorometric assay of histamine. The stability of histamine, however, was reduced in the presence of dipersulphate with BSS but not with Eagles MEM (see below).

Effect of pH, time of incubation, calcium concentration and DSCG on histamine release by persulphates

The pH profiles of histamine release for compound 48/80 and potassium and ammonium dipersulphates in BSS at 37°C for 30 min are shown in Fig. 3. The histamine release by these persulphates in BSS following an incubation time of 30 min was similar to that observed in Eagles MEM with an incubation time of 40 min.

The optimum pH for the release of histamine by both potassium and ammonium dipersulphate in BSS was 6·8 while that for compound 48/80 was 7·2. Incubation of known amounts of histamine (500–1000 ng base) with potassium or ammonium dipersulphate (2·7 mg/ml) in BSS under the conditions used to measure the pH profiles, however, resulted in 60 and 30% reductions, respectively, in the amounts of histamine recovered.

The time courses of histamine release for potassium and ammonium dipersulphate (2·7 mg/ml in Eagles MEM) are shown in Fig. 4. Compared with compound 48/80 and potassium monopersulphate, both of which produced maximum release within 5 min, the two dipersulphates released histamine only slowly.

In initial experiments on the effect of reducing the extracellular divalent cation level, addition of EDTA (3 mM) to the Eagles MEM completely inhibited the release obtained with potassium dipersulphate (2·7 mg/ml). Under similar conditions, the release obtained with compound 48/80 (0·83 μg/ml) and with ammonium dipersulphate (2·7 mg/ml) were reduced by 35 and 49% respectively. DSCG (2·7 mM) administered simultaneously with potassium dipersulphate or potassium monopersulphate to the mast-cell suspension, had no significant effect on the amount of histamine released over a 40-min incubation time.

Haemolytic action on sheep erythrocytes

Sheep erythrocytes were haemolysed by potassium monopersulphate at its acid pH or when adjusted to pH 7 with sodium hydroxide. The concentrations that caused disruption of the mast-cell membrane also caused disruption of the erythrocyte membrane. Potassium and ammonium dipersulphate had no action on sheep erythrocytes at any of the concentrations tested (0·33–2·7 mg/ml).

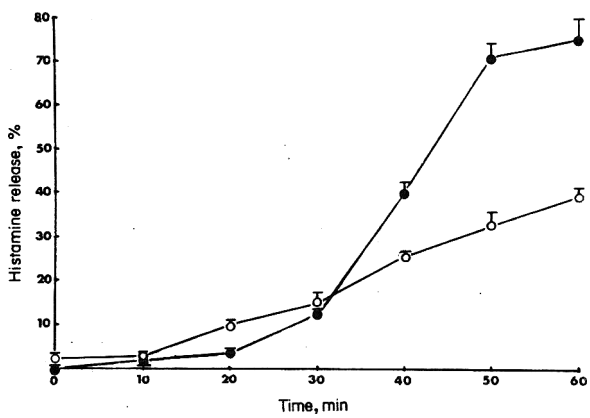

Fig. 4. Time course of histamine release by a 2·7 mg/ml concn of potassium dipersulphate (●) or of ammonium dipersulphate (○) from rat mast-cell suspensions incubated in Eagles MEM (pH 6·8) at 37°C. Each point represents the mean ± SEM (n = 10) of two separate experiments with different cell pools, all values having been corrected for appropriate spontaneous histamine release in Eagles MEM at the various time intervals.

Table 1. *Persulphate-induced histamine release from sliced guinea-pig skin in Tyrode solution*

Histamine-releasing agent	Concn (mg/0·5 ml)	Mean* histamine release (%) at 37°C	Mean* histamine release (%) at 4°C
Potassium dipersulphate	8	24·33	0·39
	4	14·73	0·22
	2	4·88	0·05
	1	3·11	0·47
	0·5	0·95	−0·75
	0·1	0·45	−0·34
Ammonium dipersulphate	8	14·42	7·59
	4	11·11	5·73
	2	6·63	3·75
	1	2·73	1·37
	0·5	2·48	−0·09
	0·1	1·11	−1·57
Potassium monopersulphate	8	23·77	15·95
	4	18·29	13·89
	2	14·41	5·01
	1	2·91	0·96
	0·5	0·13	0·12
	0·1	−0·06	0·43
Compound 48/80	0·1	10·3	−0·08

*Mean of two experiments ($n = 4$) at each temperature, results being corrected for spontaneous histamine release.

Histamine release from guinea-pig skin in vitro

The mean total histamine content of guinea-pig skin was 4·50 ± 0·14 μg base/g wet weight, with a normal mean spontaneous histamine release of 1·9 ± 0·21%. The histamine release by potassium monopersulphate and potassium and ammonium dipersulphate are shown in Table 1. All three compounds released histamine in a dose-dependent manner from guinea-pig-skin slices when incubated at 37°C for 30 min. The release elicited by potassium dipersulphate and by compound 48/80 (0·1 mg/0·5 ml) was completely inhibited by incubation at 4°C. Release by ammonium dipersulphate and potassium monopersulphate was reduced but was not completely inhibited by incubation at 4°C.

Intradermal injection of persulphates in the guinea-pig

Potassium dipersulphate produced in guinea-pig skin blue lesions which increased in size and intensity with increasing concentration. The reaction produced by this salt at 16 mg/ml (14 × 14 mm, deep blue) was similar to that caused by a standard compound 48/80 solution (80 μg/ml). The lesions produced by potassium monopersulphate, at all the concentrations tested, were of approximately the same dimensions (6 × 7 mm), but those produced by the two highest concentrations of this salt (8 and 16 mg/ml) had white centres surrounded by an area of blue.

Both the size and intensity of the skin responses produced by the persulphates and compound 48/80 were significantly reduced by pretreatment of the guinea-pigs with mepyramine. The response to a standard solution of histamine (60 μg/ml) was completely abolished by this pretreatment.

DISCUSSION

Potassium dipersulphate and monopersulphate release histamine from peritoneal mast cells and from skin, but the mechanism of histamine release by these two persulphates is apparently different. Potassium dipersulphate releases histamine from rat peritoneal mast cells by a slow, dose-dependent, non-cytolytic mechanism. By contrast, potassium monopersulphate releases histamine by a rapid cytolytic mechanism. Histamine release by ammonium dipersulphate appears to have similarities with histamine release by potassium mono- and dipersulphate. The release of histamine by compound 48/80 and potassium dipersulphate have similar characteristics, but the $ED_{40\%}$ for histamine release by potassium dipersulphate following a 40-min incubation time was approximately 4000 times greater than that by compound 48/80 on a weight basis.

Significant histamine release by potassium dipersulphate from unpurified mast cells was not observed until after incubation for approximately 30 min at 37°C. The unpurified mast-cell preparations used in these studies contained 5–10% mast cells, and their total histamine content and histamine release curves with compound 48/80 were similar to those reported by other authors (Ichikawa, Kaneko, Mori & Tomita, 1977; Johnson & Moran, 1974). Although the possibility of an interaction between the test substances and the cells other than mast cells in these preparations cannot be excluded, essentially similar results were obtained (authors' unpublished data, 1978) with mast-cell preparations purified to approximately 90% using the method of Sullivan, Parker, Stenson & Parker (1975). Using either cell preparation, the time course for histamine release was very much slower than that obtained using compound 48/80.

None of the test substances used in this study interfered in the spectrofluorometric assay of histamine. Histamine breakdown, however, was observed in the presence of dipersulphate in BSS but not in Eagles MEM. The increased stability of histamine in Eagles MEM in the presence of dipersulphate suggests that

the organic material present in this medium retards histamine breakdown. With the exception of the pH-profile determinations, all experiments were carried out in Eagles MEM, thus avoiding the breakdown of histamine during the prolonged incubation time required to determine dipersulphate-induced histamine release.

Morphological examination of mast cells after treatment with potassium dipersulphate or compound 48/80 showed degranulation of the mast cell without disruption of the cell membrane. Preliminary measurements of lactate dehydrogenase (LDH) release suggested that no significant release of this cytoplasmic enzyme had occurred. Further evidence for the selectivity of histamine release by potassium dipersulphate was obtained from the similarity in temperature dependence between this salt and compound 48/80 (Fig. 2), an optimal pH of approximately 7 (Fig. 3), and a requirement for divalent cations in the extracellular medium.

Similar experiments with potassium monopersulphate showed that the rapid histamine release obtained with this substance resulted from disruption of the mast-cell membrane. The action of this material was not confined to mast-cell membranes, however, and a similar lytic action was observed with sheep erythrocytes. Potassium and ammonium dipersulphate had no action on the sheep erythrocyte membrane, and this was consistent with their action on mast cells. However, the action of EDTA in reducing histamine release by potassium monopersulphate was not consistent with a cytolytic mechanism.

The release of histamine by the action of ammonium dipersulphate showed some of the characteristics of histamine release by potassium dipersulphate and potassium monopersulphate. Thus the release of histamine was slow, and resulted in an alteration of the mast-cell granules without membrane disruption, but the temperature dependence of histamine release did not suggest a selective mechanism. Because the ammonium ion itself can release histamine from mast cells (Charles & Menzel, 1975), apparently by a non-selective mechanism, it is possible that the histamine release observed with this compound is the result of selective and non-selective histamine release elicited by the persulphate and ammonium ion, respectively.

Disodium cromoglycate did not have a significant effect on the histamine release induced by potassium dipersulphate over a 40-min incubation time, but because the inhibition by DSCG of allergic histamine release from these cells is reversed by incubation times exceeding 5 min (Kusner, Dubnick & Herzig, 1973), it is not possible to conclude with any certainty that DSCG had any effect in these experiments. As expected, DSCG had no effect on the cytolytic release evoked by potassium monopersulphate over a 5- or 40-min incubation time.

Potassium dipersulphate, but not potassium monopersulphate or ammonium dipersulphate, also appeared to release histamine selectively from sliced guinea-pig skin. The results of these experiments confirmed earlier findings (Mahzoon *et al.* 1977) that ammonium dipersulphate at dose levels up to 1 mg/ml did not release significant amounts of histamine from guinea-pig-skin slices. Significant amounts of histamine were released, however, by higher concentrations of ammonium dipersulphate and potassium monopersulphate, although the release was depressed but not abolished by reducing the incubation time to 4°C. Although this reduction in histamine release at 4°C contradicts the findings of Mahzoon *et al.* (1977), the latter authors used rat skin and required a much lower concentration of ammonium dipersulphate to induce histamine release. Histamine release induced by potassium dipersulphate was completely inhibited by a decrease in the incubation temperature to 4°C; this mimicked the action of compound 48/80 in the same assay system.

Similarly, potassium dipersulphate differed from potassium monopersulphate in its action on guinea-pig skin *in vivo* after contact for 40 min. The skin lesions obtained with the dipersulphate salt increased in size and intensity with increasing concentration, whereas those produced by potassium monopersulphate were not dose related. Since the intensities of all of the reactions were modified by previous treatment of the guinea-pigs with mepyramine maleate, it is likely that the vascular permeability changes induced by the persulphates are due, in part, to an indirect action mediated by released histamine from skin mast cells. However, the white areas in the centre of the lesions produced by the highest concentrations of potassium monopersulphate tested did not resolve after mepyramine treatment. The possibility that these areas were produced by acid denaturation, as observed by Opie (1962) with solutions of HCl at pH 1 and 2, cannot be excluded. The pH of the potassium monopersulphate test solution (16 mg/ml) immediately prior to intradermal injection was 2·3.

The failure of mepyramine to abolish completely the skin reactions produced by the test substances (and also those produced by compound 48/80) suggests involvement of mediators other than histamine.

CONCLUSIONS AND IMPLICATIONS

Allergic contact dermatitis, chronic and generalized urticaria, and early and late onset asthmatic reactions have been reported in some individuals following occupational exposure to persulphates (of bakers and hairdressers) or the use of hair-bleach preparations containing these salts (Bonnevie, 1939; Calnan & Shuster, 1963; Forck, 1968; Foussereau & Benezra, 1970; Pepys *et al.* 1976; Sidi *et al.* 1966). Patch tests with the relevant persulphate have confirmed allergic contact dermatitis, but the mechanism of the immediate urticarial and asthmatic reactions have remained uncertain.

The results of this study suggest that the dipersulphate ion is a selective histamine-releasing agent, which slowly degranulates mast cells without disruption of the mast-cell membrane. The histamine-releasing action is confined to the dipersulphate ion; it is not observed with monopersulphate, which disrupts mast-cell and probably other cell membranes. Ammonium dipersulphate, the most frequently implicated cause of skin reactions in 'persulphate'-sensitive individuals, releases histamine by a mechanism which, to some degree, mimics the release observed with each of the two potassium salts. It seems likely that this compound has two mechanisms that contribute to hist-

amine release, namely selective release by the dipersulphate ion and non-selective release by the ammonium ion. It should be emphasized, however, that the potency of all three persulphates is considerably less than that of compound 48/80 under the same conditions, and that the original description of persulphate as a weak histamine liberator (Calnan & Shuster, 1963) is well justified.

The slow histamine release elicited from mast cells *in vitro* by potassium and ammonium dipersulphate is consistent with the observations of slowly developing scratch or intradermal skin-test responses in 'persulphate'-sensitive individuals (Calnan & Shuster, 1963). If this reaction is due to the direct histamine-releasing action of dipersulphate on mast cells, however, it is necessary to propose that persulphate-sensitive individuals either have greater numbers of skin mast cells, or have mast cells that are more labile than those in normal skin. Against this hypothesis is the failure of Calnan & Shuster (1963) to show any abnormal response in these subjects to injected compound 48/80. The failure to detect differences in the response to compound 48/80 may reflect differences in the mechanism of histamine release by this compound and by dipersulphate.

The difference between persulphate-sensitive individuals and normal subjects may lie in the presence of a delayed hypersensitivity to the persulphate ion, which may predispose these individuals to the histamine-liberating action of dipersulphate. Experiments in mice (Gershon, Askenase & Gershon, 1975) have suggested that vaso-active amine release and increased numbers of skin mast cells may play an important part in the development of delayed hypersensitivity reactions. This factor may be important in the development of skin reactions in persulphate sensitive individuals.

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COMPARISONS OF RESPONSE OF FISCHER-344 AND CHARLES RIVER RATS TO 1·5% NITRILOTRIACETIC ACID AND 2% TRISODIUM NITRILOTRIACETATE, MONOHYDRATE

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Abstract—The responses of Charles River and Fischer-344 rats to dietary 1·5% nitrilotriacetic acid and 2% trisodium nitrilotriacetate, monohydrate were compared in a 4-wk feeding study. The Charles River rats consumed more nitrilotriacetate (μmol/kg body weight/day) than the Fischer-344 rats. In spite of the different ingestion rates, the two strains of animals gave similar qualitative responses to nitrilotriacetate. The ingestion of nitrilotriacetate was associated with reduced growth, increased kidney/body weight ratio, increased urinary Ca, haematuria, and the presence of crystalline CaNaNTA in the urine.

INTRODUCTION

Charles River (CR) rats have been used in this laboratory for work on nitrilotriacetate (NTA) disposition both in long-term (Nixon, Buehler & Niewenhuis, 1972) and short-term studies (Anderson & Kanerva, 1978a,b; Michael & Wakim, 1971, 1973; Nixon, 1971). High doses of NTA (>0·75% of the diet) were found to be nephrotoxic in a 90-day feeding study (Nixon, 1971). Dietary levels of 0·15% $Na_3NTA.H_2O$ or 0·5% CaNaNTA produced hydropic degeneration of the kidneys in a 2-yr feeding study (Nixon *et al.* 1972), while 0·03% $Na_3NTA.H_2O$ had no effect. The National Cancer Institute (1977) reported that extreme doses of H_3NTA (0·75% and 1·5%) and $Na_3NTA.H_2O$ (2%) caused urinary tract cancers in Fischer-344 (F-344) rats while lower levels (<0·5%) did not produce neoplasia in the urinary tract. It has recently been shown that doses of NTA ≥ 0·75% in the diet result in the presence of crystalline CaNaNTA in the urine of CR rats (Anderson & Kanerva, 1978b) and that the dose–response of crystalluria in the CR rats was similar to the dose response of neoplasia in the F-344 rats. The present report describes an experiment comparing the urinary and kidney changes of CR and F-344 rats fed for 4 wk on levels of NTA previously reported to be carcinogenic.

EXPERIMENTAL

Fischer-344 rats of both sexes were obtained from Microbiological Associates, Laboratory Animal Division, Walkersville, MD, and were 5 to 6 wk old at shipment. The male F-344 rats weighed 84 ± 2 g (n = 30) and the females weighed 81 ± 1 g (n = 30) at the start of the test. The CR rats were weanlings obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA; the males weighed 66 ± 2 g (n = 15) and the females 59 ± 2 g (n = 14).

The $Na_3NTA.H_2O$ used in this study was a commercial sample (Monsanto Chemical Co., St. Louis, MO) and the H_3NTA was prepared by HCl precipitation from a solution of $Na_3NTA.H_2O$. The diets were physical mixtures of Chow with the appropriate weight of $Na_3NTA.H_2O$ or H_3NTA.

The animals were kept for 3 days on ground Purina Laboratory Chow and were then randomly assigned to groups receiving the control diet (Chow); 1·5% H_3NTA in Chow; or its equivalent, 2·0% $Na_3NTA.H_2O$ in Chow. Each treatment group comprised ten male and ten female F-344 and five male and five female CR animals except for the CR female control group which comprised only four animals. The animals were housed individually in stainless steel cages with wire mesh bottoms and urine and faecal separators, and kept at a controlled temperature (72° ± 2°F) and humidity (50% ± 10%) with a 12-hr light/dark cycle. All the animals were given diet and distilled water *ad lib.* Body weights and food intakes were determined weekly.

On days 4, 11, 18 and 25, the total 24-hr urine samples from each animal were collected. The volumes were recorded, the pHs were determined and the samples were inspected for the presence of blood and crystalline material.

The urines voided on day 25 by the individual CR rats and pooled samples from F-344 rats were assayed for NTA content by isotope dilution (Anderson & Kanerva, 1978a). The urine samples voided on day 18 were assayed for their total Ca content by atomic absorption spectroscopy (Anderson & Kanerva, 1978a).

On day 29 all the animals were anaesthetized with an ip injection of pentobarbitone (Nembutal, 50 mg/100 g) and both kidneys were excised and weighed.

The data were tested by analysis of variance by standard procedures* but the effects of NTA were reported as percentages of control values.

*Details of the program can be obtained from the author.

Table 1. *Comparison of Charles River and Fischer-344 rats after 4 wk on a control diet*

Sex	Strain and no. of animals	Weight gain† (g)	Feed efficiency† ([weight gain/food intake] × 100)	Kidney:body weight ratio (g/100 g)	Urine volume‡ (ml/100 g/day)
Male	CR (5)	132 ± 10	34·9 ± 1·4	0·93 ± 0·03	6·5 ± 0·8
	F-344 (10)	102 ± 2	32·8 ± 0·5	0·86 ± 0·01	3·7 ± 0·2
	(CR/F-344 ratio)	(1·29***)	(1·06)	(1·08*)	(1·76**)
Female	CR (4)	102 ± 9	30·7 ± 1·8	0·93 ± 0·04	7·6 ± 0·2
	F-344 (10)	43 ± 1	18·0 ± 0·6	0·85 ± 0·01	3·1 ± 0·1
	(CR/F-344 ratio)	(2·37**)	(1·71***)	(1·09*)	(2·46***)

†These values are calculated using data for the first 3 wk only.
‡Urine volumes were based on day 18 urine collections.
Values are means ± SEM for the numbers of animals shown. Ratios marked with asterisks indicate a statistically significant difference between the two strains: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

RESULTS

A comparison of the data for the CR and F-344 animals, consuming the control diet, is presented in Table 1. The CR rats had slightly larger kidneys and voided a much larger volume of urine/100 g body weight than the F-344 animals. Because food intake data for the F-344 rats was not available for wk 4, data on weight gain and food conversion efficiency for both strains are presented for the first 3 wk only. Although the differences between rat strains was greater in the females than in the males, the CR rats of both sexes gained more weight and were more efficient converters of diet to body weight in the 3-wk period measured. Since younger animals gain weight more rapidly and are more efficient converters of diet, differences in these measurements may be at least partially accounted for by the fact that the F-344 rats were 2–3 wk older at the beginning of the study.

Table 2 shows the effect of 1·5% H_3NTA and 2% $Na_3NTA.H_2O$ on the two strains of rats expressed as a percentage of the value for the corresponding controls. The CR rats ingested more NTA than their F-344 counterparts. This strain difference in NTA ingestion ranged from 1·6:1·00 (CR:F-344) for the females consuming 1·5% H_3NTA to 1·9:1·00 (CR:F-344) for the females consuming 2% $Na_3NTA.H_2O$. Both forms of NTA reduced weight-gain in all groups and in most instances the growth reduction was greater in the F-344 animals than in the CR rats although the latter were consuming at least 1·6 times as much NTA as the F-344 rats. The inclusion of 2% $Na_3NTA.H_2O$ resulted in reduced food conversion efficiency in the males but not in the females. Food conversion efficiencies of the CR rats were not influenced by dietary H_3NTA but in the F-344 animals H_3NTA caused a statistically significant reduction in efficiency.

Ingestion of $Na_3NTA.H_2O$ increased kidney:body weight ratios in all groups but the response was most marked in the male, CR animals (150% increase). In contrast, H_3NTA resulted in minimal increases in kidney:body weight ratios (3–11%). Urine volume/100 g body weight was elevated by $Na_3NTA.H_2O$ ingestion, particularly in the male CR animals, but the response was variable and did not simply reflect $Na_3NTA.H_2O$ ingestion rate. In contrast, H_3NTA reduced urine volumes in the male rats and had no consistent effect in the female animals.

Table 3 summarizes some additional urinary observations that were influenced by NTA ingestion. The two forms of NTA produced consistent but opposite responses in urinary pH; $Na_3NTA.H_2O$ ingestion resulted in a pH increase and H_3NTA ingestion reduced the urinary pH in all cases. Although the percentage of male F-344 animals showing a haematuria on day 18 was the same for both forms of NTA, the percentage in all the other groups was greater when $Na_3NTA.H_2O$ was fed than when H_3NTA was fed. All of the animals ingesting the NTA had crystalline material in their urines and in all instances this material yielded an X-ray diffraction pattern identical to that of pure CaNaNTA. Visual assessment suggested that the amount of crystalline material varied between groups but no attempt was made to quantitate the crystalline mass.

The urinary concentrations of Ca varied considerably between treatments but in all instances NTA ingestion resulted in a marked increase in the percentage of the ingested Ca that was recovered in the urine (data not presented). The urinary NTA concentrations showed a two-fold variation but when expressed as a percentage of the ingested dose the values were in the 30–40% range for all groups.

Only one F-344 animal developed hydronephrosis during the study, compared with 100% of the male CR and 40% of the female CR rats receiving $Na_3NTA.H_2O$ and 60% of the male CR animals receiving H_3NTA.

DISCUSSION

Comparison of the response of CR and F-344 rats to diets containing 2% $Na_3NTA.H_2O$ and 1·5% H_3NTA for 4 wk shows that there is little qualitative difference in response between the two strains. For example, both strains develop crystalluria and haematuria (Table 3) although their NTA ingestion rates are quite different when expressed as μmol/kg body weight/day (Table 2). This similarity in response to different loads is more understandable in view of the similar urine concentrations of Ca and NTA of the two strains. It should be noted that the reported concentrations are based upon total 24-hr urine samples and may underestimate the peak concentrations of both Ca and NTA in the urine. For example, we have noted that morning urines (after night ingestion) con-

Table 2. *Comparison of the effects on Charles River and Fischer-344 rats of feeding diets containing 2% $Na_3NTA.H_2O$ or 1·5% H_3NTA for 4 wk*

Treatment	Sex	Strain and no. of animals	NTA intake (μmol/kg/day)	% of corresponding control value			
				Weight gain*	Food conversion efficiency	Kidney:body weight ratio (g/100 g)	Urine volume (ml/100 g/day)
2% $Na_3NTA.H_2O$	Male	CR (5)	8·9	63*	86	252***	249**
		F-344 (10)	5·1	58***	81**	126**	106
	Female	CR (5)	9·5	86	96	120**	134*
		F-344 (10)	5·1	86	105	114***	145***
1·5% H_3NTA	Male	CR (5)	8·9	89	103	109	81
		F-344 (10)	5·5	64***	87**	108**	69***
	Female	CR (5)	9·2	81	102	103	113
		F-344 (10)	5·9	70***	83*	111***	97

Values marked with asterisks show a statistically significant difference from the control values (*$P < 0·05$; **$P < 0·01$; ***$P < 0·001$).

Table 3. *Comparison of urine characteristics and incidence of hydronephrosis in Charles River and Fischer-344 rats after 4 wk on diets containing 1·5% H_3NTA or 2% $Na_3NTA.H_2O$*

Sex	Strain	Treatment and no. of animals	Urine parameter					Hydronephrosis (%)
			pH	Hb† (90)	Crystals‡	Ca (μmol/ml)	NTA (μmol/ml)	
Male	CR	Control (5)	7·1 ± 0·1	0	−	5 ± 1	0	0
		H_3NTA (5)	6·1 ± 0·1	20	+	23 ± 2***	45 ± 2	60
		$Na_3NTA.H_2O$	8·4 ± 0	100	+	6 ± 2	18 ± 5	100
	F-344	Control (10)	6·8 ± 0	0	−	10 ± 1	0	0
		H_3NTA (10)	6·2 ± 0	80	+	23 ± 2**	26	0
		$Na_3NTA.H_2O$ (10)	8·4 ± 0	80	+	15 ± 2*	33	10
Female	CR	Control (4)	7·0 ± 0·1	0	−	6 ± 2	0	0
		H_3NTA (5)	6·5 ± 0·1	0	+	23 ± 4*	34 ± 4	0
		$Na_3NTA.H_2O$ (5)	8·3 ± 0·1	40	+	14 ± 1*	23 ± 6	40
	F-344	Control (10)	7·0 ± 0·1	0	−	7 ± 1	0	0
		H_3NTA (10)	6·3 ± 0·1	20	+	18 ± 1***	24	0
		$Na_3NTA.H_2O$ (10)	8·7 ± 0·1	70	+	17 ± 1***	37	0

†Percentage of urine samples containing visible blood on day 18.
‡Presence of visible crystalline material in urine on day 18.
§Ca in 24-hr urine sample collected on day 18.
‖NTA in 24-hr urine samples collected on day 25.
¶Incidence of hydronephrosis noted on day 28 when animals were killed.
Values are means ± SEM for the number of animals shown (except for the urine NTA values of the F-344 rats which were based on a pooled sample), and those marked with asterisks differ significantly from the control values (*$P < 0·05$; **$P < 0·01$; ***$P < 0·001$).

tains a greater density of crystalline CaNaNTA than afternoon urines. In fact afternoon urines were frequently devoid of visible crystals. In both strains of rats the only crystalline material detected by X-ray diffraction was CaNaNTA.

The one parameter that showed a clear strain difference was the effect of 2% $Na_3NTA.H_2O$ on the incidence of hydronephrosis—this response was far greater in CR rats than in F-344 rats. This strain difference may be a consequence of the difference in NTA ingestion rate (Table 2). Further, the development of hydronephrosis in the F-344 rats as a result of ingesting 2% $Na_3NTA.H_2O$ might have been apparent had the feeding been continued for a longer period since this response was noted in almost all F-344 rats given 2% $Na_3NTA.H_2O$ for 2 yr in the NCI bioassay (National Cancer Institute, 1977).

Overall, the results demonstrate that the responses in two strains of rats to dietary NTA are similar and that the conditions noted in CR rats receiving a high dose of NTA (Anderson *et al.* 1978a; Michael *et al.* 1971) are also produced in F-344 rats by the same dietary concentrations of NTA although these dietary concentrations result in different ingestion rates and systemic loads of NTA in the two strains.

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SHORT PAPERS

DETECTION OF MUTAGENIC POLYCYCLIC AROMATIC HYDROCARBONS IN AFRICAN SMOKED FISH

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Abstract—The mutagenicity of six polycyclic aromatic hydrocarbons found in African smoked fish was tested using several *Salmonella typhimurium* strains. In the presence of fortified rat-liver post-mitochondrial fractions, mutagenic activity was observed with *o*-phenylenepyrene, coronene, benzo[*g,h,i*]-perylene and triphenylene in the plate incorporation method, and with fluoranthene in the bacterial fluctuation test. No mutagenic effects of benzo[*b*]fluoranthene towards any of the tested strains were detected.

Introduction

The high incidence of stomach cancer among populations living in Iceland and in Baltic countries has been causally related to the intensive consumption of smoked fishes by people from those countries (Bailey & Dunjal, 1958). Various polycyclic aromatic hydrocarbons (PAHs) have been isolated from industrially smoked fish (Bailey & Dunjal, 1958; Grimmer & Hildebrandt, 1967; Howard, Teague, White & Fry, 1966; Lijinsky & Shubik, 1965). However, wood-smoked fish were less extensively investigated although comparatively high levels of benzo[*a*]pyrene (37 μg/kg) have been detected in such fish (Masuda & Kuratsune, 1971). Many PAHs that are carcinogenic in rats have been isolated from cigarette smoke condensates (Akin, Snook, Severson, Chamberlain & Walters, 1976); moreover, carcinogenicity and mutagenicity tests have been carried out on the various fractions obtained after chromatography of such condensates on a silica gel column (Bock, Swain & Stedman, 1970; Kier, Yamasaki & Ames, 1974). Recently, the skin from smoked fishes, smoked meats and the smoke condensate used for their processing (Nagao, Honda, Seino, Yahagi & Sugimura, 1977) were shown to be mutagenic towards *Salmonella typhimurium*.

For a large proportion of people from Central Africa, an essential part of their dietary protein is obtained from fish smoked by an artisanal wood combustion procedure. Twenty PAHs have been isolated and identified in smoked fish from Zaire (K. Mossanda, unpublished data, 1978). Some of these are reported to exert various carcinogenic (C) effects in animals (Dipple, 1976; Hoffmann & Wynder, 1976) and, or mutagenic (M) effects towards *S. typhimurium* strains (McCann, Choi, Yamasaki & Ames, 1975) as indicated below: anthracene (M), phenanthrene (M), fluoranthene, pyrene (M), chrysene (C,M), benz-[*a*]anthracene (C,M), triphenylene, benzo[*a*]pyrene (C,M), benzo[*b*]fluoranthene (C), benzo[*j*]fluoranthene (C), perylene, benzo [*e*]pyrene (C,M), benzo-[*g,h,i*]perylene (C), *o*-phenylenepyrene, anthanthrene, coronene, dibenz[*a,c*]anthracene (C,M), benzo[*b*]-tetraphene (C,M), dibenz[*a,h*]anthracene (C,M), dibenzo[*a,l*]pyrene (C,M). Since the mutagenic activity of some of these compounds was still unknown, six compounds were tested using the *S. typhimurium* strains. The compounds tested were: coronene, benzo[*g,h,i*]perylene, triphenylene, fluoranthene, *o*-phenylenepyrene and benzo[*b*]fluoranthene.

Experimental

Materials. Coronene and benzo[*g,h,i*]perylene were obtained from Fluka (Leuven); triphenylene, fluoranthene and *o*-phenylenepyrene from Aldrich Europe (Beerse), and benzo[*b*]fluoranthene from Koch-Light Laboratories Ltd. (Brussels). All other products were of the purest grade commercially available. PAHs were stored in the dark at 4°C in the presence of gaseous nitrogen. Dilutions were made in dimethylsulphoxide (DMSO).

Animals. Adult male Wistar rats (200–250 g) were fed an RAL diet. The animals were injected ip (dosage; 500 mg/kg) with Arochlor 1254 diluted in corn oil (200 mg/ml) 5 days before the preparation of the liver fractions (Ames, McCann & Yamasaki, 1975).

Mutagenicity assays. S. typhimurium strains TA1530, TA1535, TA1537, TA1538, TA98 and TA100 were kindly provided by Professor B. N. Ames. The post-mitochondrial (S9) fractions were obtained from three pooled rat livers, the homogenate (3 ml of 0·15 M-KCl/g wet liver) of which was centrifuged by the classical procedure (Ames *et al.* 1975). The S9 mix was prepared according to Ames *et al.* (1975) by adding $MgCl_2$ (8 μmol/ml mix), KCl (33 μmol/ml mix), sodium phosphate (100 μmol/ml mix), glucose-6-phosphate (5 μmol/ml mix) and $NADP^+$ (4 μmol/ml mix). Either 100 μl (25 mg wet liver)/ml or 300 μl (75 mg wet liver)/ml mix of S9 were used.

Plate tests were performed in duplicate by mixing substrate dilutions (0·1 ml/plate), 2–8 × 10^7 bacteria

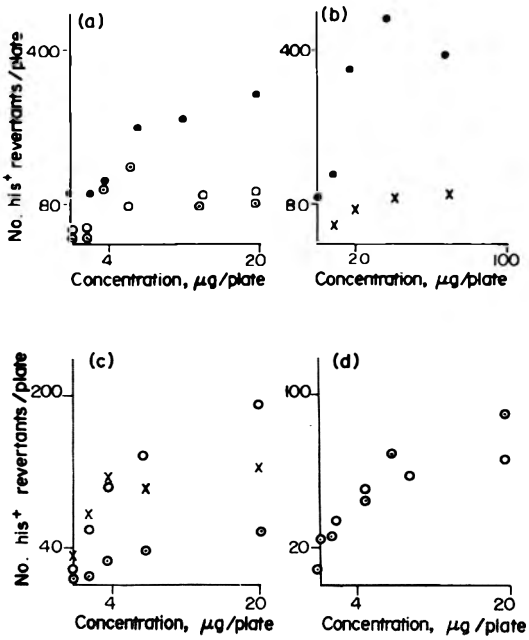

Fig. 1. Mutagenic activity of PAHs towards *S. typhimurium* strains TA1538 (○), TA100 (●), TA1537 (⊙), TA98 (×) in the presence of S9 mix Arochlor 1254 (100 μl S9/ml mix); (a) *o*-phenylenepyrene, (b) triphenylene, (c) coronene, (d) benzo[*g,h,i*]perylene.

from an overnight culture in nutrient broth (Difco)/plate, and S9 mix (0·5 ml/plate) in histidine–biotin (0·05 mM)-supplemented top agar (2 ml/plate), which was layered on minimal glucose agar (Vogel Bonner E medium) in Petri dishes. The plates were incubated for 48 hr at 37°C in the dark and the numbers of *his*$^+$ revertant colonies were calculated. The toxicity of the substrate was evaluated by determining the bacterial survival with a lower bacterial inoculum (10^4-fold dilution) and plates of nutrient agar (Difco).

Bacterial fluctuation tests were performed in triplicate using a modification of the method proposed by Green, Bridges, Rogers, Horspool, Muriel, Bridges & Fry (1977). Into a sterile tube kept in iced water, were successively introduced: liquid minimal glucose medium (Vogel-Bonner E medium) supplemented with histidine and biotin (0·005 mM) (4 ml), 2–8 × 10^7 bacteria from an overnight culture in nutrient broth, substrate dilution in DMSO (0·1 ml), and S9 mix the composition of which was 300 μl S9/ml mix (1 ml). After homogenization, the mixture was distributed at a constant volume of 0·1 ml/tube in 50 sterile tubes. The tubes were then incubated at 37°C in the dark for 3 hr. Histidine–biotin-supplemented liquid minimal glucose medium (2 ml) containing bromocresol purple (BCP) (5 μg/ml) was added to each tube. The incubation was then carried on at 37°C until a total incubation time of 72 hr had elapsed. The numbers of growing positive tubes/rack were counted.

Results

No mutagenic effect was detected with benzo[*b*]fluoranthene towards any of the tested strains. In the presence of S9 mix, mutagenic ictivity was observed with *o*-phenylenepyrene, coro ene, benzo[*g,h,i*]perylene and triphenylene by the plate incorporation method (Fig. 1). Under these conditions, no cytotoxic effects of PAHs were detected. With o-phenylenepyrene, the strains TA100, TA1537 and TA1538 reverted at concentrations in the region of 3 μg/plate. TA100 *his*$^+$ revertants increased considerably with triphenylene at 10 μg/plate or more. With coronene,

Table 1. *Results of fluctuation tests*

Polycyclic aromatic hydrocarbon	Strain	Substrate dilution (μg/ml)	Average no. of positive tubes per rack		Significance (P)
			Control	Treated	
Benzo[*b*]fluoranthene	TA98	10	21	20·3	NS
		100		18	NS
	TA100	10	23·6	24·6	NS
		100		30·6	NS
Triphenylene	TA98	50	18·3	26·6	NS
		100		30·3	<0·05
	TA100	50	36·3	36	NS
		100		41·3	NS
Benzo[*g,h,i*]perylene	TA98	1	17·3	21·3	NS
		10		27	<0·05
	TA100	1	33	33·6	NS
		10		39·3	NS
	TA1538	1	19·6	15	NS
		10		31·3	<0·05
Coronene	TA98	2	26·6	32·6	NS
		20		38	<0·05
	TA100	2	18·3	18·3	NS
		20		19·3	NS
o-Phenylenepyrene	TA98	0·5	23	31·6	NS
		5		32	NS
Fluoranthene	TA98	10	18·3	22·6	NS
		100		46·3	<0·01
	TA100	10	29·3.	26·3	NS
		100		50	<0·01

NS = Not significant

TA1538 and TA98 were the most sensitive strains: a reversion to histidine prototrophy was noticeable at a concentration of 1 μg/plate. With benzo[g,h,i]-perylene, a concentration of 2 μg/plate caused the strain TA1537 to revert.

By utilizing the bacterial fluctuation test (Table 1), weakly positive effects were observed with triphenylene (TA98), benzo[*g,h,i*]perylene (TA98, TA1538) and coronene (TA98). With fluoranthene, a highly significant positive response ($P < 0{\cdot}01$) was obtained with the strains TA98 and TA100 although this latter compound showed no mutagenic activity in the classical plate incorporation method.

Discussion

Because of their poor water solubility, these PAHs were generally tested at a narrow range of concentrations. Nevertheless, the doses where an increasing reversion to prototrophy was observed (1–10 μg/plate) were of the same order as the doses of other common PAHs such as benzo[*a*]pyrene that are active. Moreover, the strains sensitive towards these compounds were those on which many PAHs have already demonstrated a positive mutagenic effect (McCann *et al.* 1975). These results point out the possible long-term deleterious effects associated with the presence of PAHs in the human environment and particularly in some smoked foods.

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INHIBITORY ACTION OF THE MYCOTOXINS PATULIN AND PENICILLIC ACID ON UREASE

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Abstract—Patulin and penicillic acid inhibited the activity of the enzyme urease. The inhibitory action was reduced by addition of cysteine, suggesting that the toxin–enzyme interaction was due to a blocking of essential thiol groups in the enzyme molecule. The lowest concentrations of patulin and penicillic acid found to have an inhibitory effect on the urease preparation tested were $1{\cdot}7 \times 10^{-5}$ M and $1{\cdot}1 \times 10^{-5}$ M, respectively.

Introduction

Patulin is a toxic secondary metabolite of *Penicillium patulum*, *P. expansum* and other moulds (Reiss, 1972) and is known to interact with compounds containing thiol groups, such as cysteine and glutathione (Hoffmann, Mintzlaff, Alperden & Leistner, 1971; Reiss, 1976). The patulin–cysteine adducts retain some bacteriostatic activity (Geiger & Conn, 1945) and are teratogenic to chick embryos (Ciegler, Beckwith & Jackson, 1976). Various enzymes with essential thiol groups, such as lactic and alcoholic dehydrogenases (Ashoor & Chu, 1973a) and muscle aldolase (Ashmoor & Chu, 1973b) are inhibited by patulin. It was recently shown (Reiss, 1977) that this toxin reduces the activity of urease, which has thiol groups as its active sites (Gorin, 1962).

Penicillic acid is a toxic and carcinogenic metabolite of various Penicillium species (Reiss, 1972). According to Ciegler, Mintzlaff, Weisleder & Leistner (1972) it is capable of reacting with thiol-containing amino acids. The resulting adducts are not toxic to mice and quails but are still toxic to the chick embryo.

Since it was not known whether penicillic acid reduced the activity of the thiol-enzyme urease, the influence of this mycotoxin and also of patulin on the activity of urease was studied quantitatively for purposes of comparison. As cysteine, an amino acid containing a thiol group, is able to react with patulin as well as with penicillic acid (Ciegler *et al.* 1972) it seemed to be possible that the inhibitory action of these toxins towards urease might be reduced by this amino acid.

Experimental

Influence of the toxins on urease. Urease (Merck AG, Darmstadt) and urea (Merck AG), each in 1% aqueous solution, were mixed in volumes of 1 and 20 ml, respectively, and the conductivity of the mixture was measured at 1-min intervals with a recorder ENV 4 (Technowa, Neuss). After the fifth measurement, 1 ml of a 0·02% aqueous solution of patulin (Dr. F. A. Norstadt, Agricultural Research Service, Fort Collins, Colorado, USA) or of penicillic acid (Makor Chemicals Ltd., Jerusalem, Israel) was added and the conductivity was recorded for another 6 min.

Influence of cysteine on the action of the toxins. The enzyme test was performed as described above, and 6 min after the addition of patulin or penicillic acid, 3 ml of a 0·9% aqueous solution of cysteine (Merck AG) was added and the conductivity was recorded for another 5 min.

Determination of the lowest inhibitory concentration of the toxins. After an incubation period of 6 min the standard urea–urease mixture was mixed with 0·1–0·5 ml of the 0·02% aqueous solutions of the individual toxins and the conductivity was again recorded for 6 min.

Results

Before the addition of the mycotoxins, a constant increase in conductivity was observed in the urea–urease mixture. After the addition of penicillic acid or patulin, the increase in conductivity was markedly weaker, but additions of cysteine resulted in a further increase in the conductivity in the case of both toxins (Fig. 1). The lowest toxin levels that showed an inhibitory effect were 0·25 ml of the 0·02% patulin solution, equivalent to $1{\cdot}7 \times 10^{-5}$ M, and 0·20 ml of the penicillic acid solution, equivalent to $1{\cdot}1 \times 10^{-5}$ M (Fig. 2).

Discussion

The present study shows that penicillic acid may be an enzyme poison reacting with essential thiol groups. In this respect, the action of penicillic acid is similar to that of patulin; the ability of the latter to interact with the thiol groups of urease (Reiss, 1977) has been confirmed quantitatively. As is shown in Fig. 1, cysteine interferes with the inhibitory action of both toxins, resulting in a nearly normal increase of conductivity. The interactions of patulin and peni-

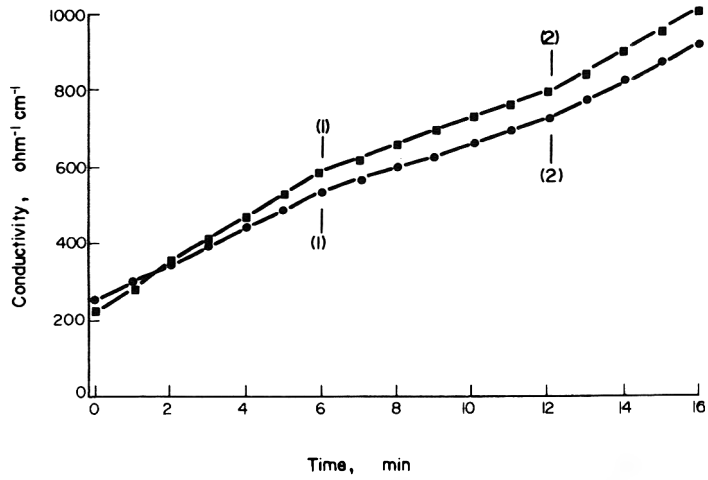

Fig. 1. Inhibition of urease by addition of 1 ml of 0·02% patulin (●) or of 0·02% penicillic acid (■) to 21 ml reaction medium, and the effect of adding 3 ml of a 0·9% solution of cysteine: (1) addition of toxin; (2) addition of cysteine.

cillic acid with cysteine, used as a model substance for thiol compounds, are based upon addition of the amino acid to the isolated double bonds of the toxin molecules (Ciegler *et al.* 1972). The lowest inhibitory concentrations in the urea–urease mixture were $1{\cdot}7 \times 10^{-5}$ M for patulin and $1{\cdot}1 \times 10^{-5}$ M for penicillic acid. Because of a lack of exact data in their paper, it cannot be explained why Hoffmann-Ostenhof & Lee (1946) came to the conclusion that patulin was unable to inhibit the activity of urease. Probably the enzyme activity in the raw urease preparation made from soya-bean meal had unusual properties.

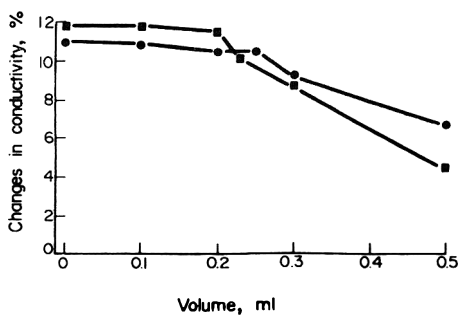

Fig. 2. Effects of various additions of patulin (●) and penicillic acid (■) on the activity of urease, as indicated by changes in conductivity (in %) recorded 5 min after addition of the toxin.

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MYCOPHENOLIC ACID IN MARKETED CHEESES

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Abstract—Analysis of a total of 100 samples of blue-moulded cheese showed that 38 contained mycophenolic acid, three at levels higher than 10 ppm. This contamination is particularly frequent in some types of cheese, and is due to the use, in their manufacture, of those *Penicillium roqueforti* strains that produce a high yield of mycophenolic acid *in vitro*.

Introduction

It has been suggested that mycophenolic acid was probably the first antibiotic substance to be purified (Wilson, 1971). Its biosynthesis has been studied in Penicillia of the *asymmetrica-velutina* type, *P. brevicompactum*, *P. stoloniferum* and *P. bialowiezense*, as well as in an *asymmetrica-fasciculata* species, *P. viridicatum* (Burton, 1949; Florey, Jennings, Gilliver & Sanders, 1946: Gosio, 1896). Recently, it was reported that cultures of various strains of *P. roqueforti* isolated from cheeses produced mycophenolic acid when grown *in vitro* on a synthetic or semi-synthetic medium or on curdled milk (Lafont, Debeaupuis, Gaillardin & Payen, 1978). This observation led us to study whether blue-moulded cheeses were contaminated with this substance.

Experimental

Cheeses. All samples came from commercial sources, and were of a degree of ripeness suitable for consumption. Analyses were carried out on various commercially marketed brands of each of the types of cheese listed in Table 1.

Extraction of mycophenolic acid. Cheese samples (20 g) were homogenized in 100 ml 5% NaCl in a Braun MX 32 homogenizer. The homogenate was adjusted to pH 6·0 with 10% acetic acid, and 100 ml methanol and 60 ml acetone were added. The mixture was homogenized again for 1 min and filtered on Fioroni 101 paper. The filtrate was stored overnight at −22°C, and then filtered on Whatman 2V paper. Acetone and some of the methanol were evaporated under vacuum, and the remaining filtrate was washed three times with an equal volume of hexane. Residual methanol was removed by evaporation, and the aqueous phase was extracted three times with an equal volume of chloroform. The chloroform solution was filtered on anhydrous sodium sulphate and concentrated under vacuum to 2 ml.

This extract was purified by partition chromatography on a 20 g Davison 100–200 mesh silica column saturated with aqueous methanol (9:1, v/v). The first elution was with 100 ml hexane equilibrated with aqueous methanol, and the second with 100 ml chloroform–methanol–hexane–acetic acid (97:2:2:0·1, by vol.).

Thin-layer chromatography and assay. Silica-gel thin-layer chromatography was carried out in jars in a saturated atmosphere, with elution by either chloroform–ethyl ether–90% formic acid (60:20:0·1, by vol.), or chloroform–ethyl acetate–90% formic acid (60:40:0·2, by vol.).

Mycophenolic acid was assayed after the drying of the chromatograms (at 120°C for 15 min), or after

Table 1. *Contamination of 100 cheese samples by mycophenolic acid*

Type of cheese	No. of samples analysed	No. of samples contaminated with mycophenolic acid at a level (ppm) between: 0·01 and 0·1	0·1 and 1	1 and 5	5 and 10	10 and 15
Gorgonzola	12	3	0	0	0	0
German blue cheese	12	2	1	0	0	0
Tilsitt	6	0	0	0	0	0
Stilton	5	0	0	0	0	0
Roquefort	25	1	2	10	5	3
Bleu des Causses	6	1	2	0	0	0
Bleu d'Auvergne	8	0	0	0	0	0
Fourme d'Ambert	9	0	0	0	0	0
Industrial French cheeses*	15	1	3	2	0	0
Melted cheese (Roquefort)	2	0	0	2	0	0

*Brand-name products of unspecified origin.

their exposure to ammonia vapour. The two assay systems used were a visual comparison of the intensity of the fluorescence with that of a known standard (crystallized mycophenolic acid, prepared in the laboratory from cultures of *P. brevi-compactum*), and fluorodensitometry using a Vernon fluorodensitometer, model PHI 5, with a high-pressure mercury source (excitation wavelength, 254 nm; secondary filter, Wratten-Kodak 47 B).

P. roqueforti *strains.* For some cheeses, cultures were obtained on Czapek medium by inoculating the mould taken from the centre of the mass. The *in vitro* capacity for mycophenolic acid production by these cultured strains was assayed in subcultures after incubation for 10 days at 15°C, according to the experimental protocol already described (Lafont *et al.* 1978).

Results

Of the 100 samples of cheese studied, 38 were found to contain mycophenolic acid. The proportion of samples so contaminated varied with the type of cheese. The Roquefort samples showed the highest incidence, with 84% affected, while contamination was rare in other types. None was detected in the Bleu d'Auvergne, Fourme d'Ambert, Tilsitt and Stilton samples (Table 1). High concentrations of mycophenolic acid were found only in the Roquefort samples, the maximum being 14·3 ppm.

All of the strains of blue mould isolated from 34 samples of cheese were found to belong to the species *P. roqueforti* Thom, according to the description of Samson, Eckardt & Orth (1977). Mycophenolic acid was assayed in chloroform extracts of these cultures, after 10 days at 15°C on modified Czapek medium (Brian, Dawkins, Grove, Heming, Love & Norris, 1961). The results are shown in Table 2. A close correlation is apparent between the mycophenolic acid levels in a cheese sample and in the culture derived from the same sample. In no case did cultures obtained from cheeses in which no mycophenolic acid was found produce more than 0·040 mg mycophenolic acid/g dry weight of culture. In contrast, all cultures producing more than 1 mg/g came from cheeses with mycophenolic acid levels greater than 1 ppm.

Discussion

The analytical technique that we have developed is relatively simple and fast. Its sensitivity is satisfactory, quantities as small as 0·002 μg being detected while concentrations in cheese as low as 0·02 ppm can be determined quantitatively. Addition of mycophenolic acid to samples has shown that recovery is between 80 and 85%. This method yields extracts less rich in pigments and lipids than those produced by the method of Shih & Marth (1971) and used by Scott & Kennedy (1976) to assay for roquefortine in cheeses.

Mycophenolic acid is better known for its antibiotic action, against bacteria and micromycetes, and for its antiviral properties than for its toxicity. Nevertheless, Wilson (1971) classed it among the mycotoxins, citing studies in the rat, monkey and other species. In the rat, oral administration of daily doses of the order of 30 mg/kg caused anaemia, with death following several weeks later. Lower doses fed to female rats prevented implantation of fertilized ova. Monkeys fed 150 mg/kg daily for 2 wk developed abdominal pains, diarrhoea with bleeding and anaemia. Chick-embryo development was inhibited by relatively small doses of mycophenolic acid, the LD_{50} being approximately 1 μg/egg (Lafont *et al.* 1978). Long-term toxicity studies on rabbits, however, have demonstrated that daily oral doses of 80 or 320 mg/kg do not produce pathological changes (Adams, Todd & Gibson, 1975), and in mice the LD_{50} is 973 mg/kg by the ip route and higher than 1·25 g/kg by oral administration (Williams, Boeck, Cline, De Long, Gerzon, Gordee, Gorman, Holmes, Larsen, Lively & Matthews, 1968). From the biochemical point of view, mycophenolic acid inhibits nucleic acid synthesis, exerting a specific effect upon guanine metabolism (Franklin & Cook, 1969; Kleinschmidt, Murphy & Hayes, 1977; Sweeney, Hoffman & Esterman, 1972).

It is unlikely that the consumption of contaminated cheese would cause acute toxicity, in view of the relatively low concentrations of mycophenolic acid involved. However, given the biochemical and antibiotic properties of this compound, it is desirable that commercial dairy foods consumed in large quantities should not be contaminated at levels of the order found in some samples.

Table 2. *Relation between the mycophenolic acid level in cheese and* in vitro *production of the metabolite by the* P. roqueforti *isolates*

Total no. of cheese samples	Levels (ppm) of mycophenolic acid in cheeses	No. of *P. roqueforti* isolates cultured	Mycophenolic acid (mg/g*) produced *in vitro* by individual isolates			
15	<0·01	15	0·006	0·020	0·012	0·016
			0·022	0·014	0·005	0·004
			0·009	0·040	0·005	0·024
			0·018	0·018	0·008	
4	0·01–0·1	4	0·096	0·066	0·032	0·280
4	0·1–1	5	0·880	0·240	0·310	0·460
			0·098	2·820		
4	1–5	6	0·360	0·920	1·240	2·220
			0·850			
2	5–10	2	6·250	3·080		
2	10–15	2	3·120	4·160		

*As mg mycophenolic acid/g dry culture.

Our results show that mycophenolic acid contamination of cheeses is due to the use of certain strains of *P. roqueforti* in their manufacture. From the public health viewpoint, it seems reasonable to suggest that only strains with known mycophenolic acid-synthesis levels should be used in the manufacture of cheese, a restriction most unlikely to prejudice the palatability of the products.

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THE 'CARRY-OVER' OF AFLATOXIN, OCHRATOXIN AND ZEARALENONE FROM NATURALLY CONTAMINATED FEED TO TISSUES, URINE AND MILK OF DAIRY COWS

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Abstract—Concentrate rations containing 385–1925 μg zearalenone/kg or 317–1125 μg ochratoxin A/kg were prepared from naturally contaminated cerals and were each fed to two adult cows, the zearalenone ration for 7 wk and the ochratoxin ration for 11 wk before slaughter. Aflatoxin B_1 was fortuitously present in both rations at a concentration of 20 μg/kg. Residues of zearalenone, ochratoxin α and aflatoxin B_1 were not detected in muscle, liver, kidney, serum, milk or urine, but ochratoxin A was detected in the kidneys of one cow at a concentration of about 5 μg/kg and aflatoxin M_1 was detected in the kidneys, milk and urine of all animals at concentrations varying from trace amounts to 0·6 μg/kg.

Introduction

During the past few years trace amounts of various fungal toxins have been detected in about 11% of samples of animal feedstuffs analysed in the course of investigational work at the Central Veterinary Laboratory, Weybridge. The most frequently encountered have been aflatoxin, ochratoxin A and zearalenone. The experimental 'carry-over' of aflatoxin B_1 or its metabolite M_1 to tissues, eggs and milk of food-producing animals has been well described by Rodricks & Stoloff (1977) and others, but milk is probably the only human food of animal origin known to be contaminated naturally with an aflatoxin. Few milk surveys have been published however (see review by Patterson, 1979), and there is little or no information concerning ochratoxin A or zearalenone residues. Therefore it seemed important to find out whether these toxins, occurring naturally in animal feeds, might pass into bovine meat or milk and present a hazard to human health.

Experimental

Naturally moulded wheat containing zearalenone, and barley containing ochratoxin A were obtained from separate farms with feed-storage problems. The cereals were ground separately and mixed with other feed components to provide balanced and palatable rations. To avoid digestive upsets, the amount of cereal was increased gradually over a period of 2 wk to a maximum of approximately 80% of the total concentrate ration in each case.

Four batches of each ration were prepared and the final mixes contained 385–1925 μg zearalenone/kg or 317–1125 μg ochratoxin A/kg. All batches of both mixes were found to contain aflatoxin B_1 at a concentration of 20 μg/kg presumably as a result of the use of a contaminated 'balancer' in formulating the final rations. Feeds were stored in a closed barn during the course of the experiment and the levels of the three mycotoxins were checked every 3–4 wk to detect any fluctuation.

Two adult Jersey milking cows were fed individually 10 kg daily of the concentrate ration containing zearalenone for 7 wk and a second pair were fed the same quantity of the ochratoxin A ration for 11 wk. Each cow received, in addition, 9 kg hay/day and water *ad lib.* The milk yield was recorded twice daily and milk samples were collected during the last week of the feeding period. Animals were weighed and bled every 14 days. Milk and serum were stored at −20°C until examined. When each feed had been exhausted, the cows were slaughtered and samples of liver, kidney, muscle (m. biceps femoris) and urine were obtained and stored at −20°C.

Aflatoxin B_1 and M_1 concentrations were determined in homogenized tissues, serum, milk and urine by an adaptation of the Romer (1975) method described by Patterson, Shreeve & Roberts (1979). Analytical limits were 0·01 μg/kg for urine and muscle, 0·02 μg/kg for serum, 0·03 μg/kg for milk and 0·05 μg/kg for kidney and liver, but smaller concentrations could be detected qualitatively.

Ochratoxin A and α were determined in milk, tissues, serum and urine using the method of Nesheim, Hardin, Francis & Langham (1973) as used by Krogh, Axelsen, Elling, Gyrd-Hansen, Hald, Hyldgaard-Jensen, Larsen, Madsen, Mortensen, Moller, Petersen, Ravnskov, Rostgaard & Aalund (1974), and zearalenone was determined by a modification of the method of Mirocha, Schauerhamer & Pathre (1974) described by Patterson *et al.* (1979).

Results and Discussion

Cows remained clinically normal throughout the feeding period and there was no obvious effect on the milk yields. At autopsy, multiple small grey lesions were found on the kidneys of both cows fed the ochratoxin-contaminated diet; histological examination showed the presence of a subacute interstitial nephritis thought to be unconnected with the ingestion of ochratoxin. No other gross lesions were seen.

Table 1. *Aflatoxin M_1 in kidneys and body fluids of cows fed diets containing zearalenone and aflatoxin B_1 or ochratoxin A and aflatoxin B_1*

Diet*	Cow no.	Aflatoxin M_1 concn (μg/kg) in Kidney	Milk	Urine
Zearalenone	1	0·07	0·06	0·50
	2	Trace	Trace	0·12
Ochratoxin A	3	0·2	0·06	0·09
	4	0·2	0·06	0·22

*The diets contained either 385–1925 μg zearalenone/kg and 20 μg aflatoxin B_1/kg or 317–1125 μg ochratoxin A/kg and 20 μg aflatoxin B_1/kg.

No residues of zearalenone (<4 μg/kg) were detected although concentrations up to nearly 2 mg/kg were present in the experimental feed. Ochratoxin A was detected only in the kidneys of one animal and the metabolite ochratoxin α was not detected at all (detection limit about 5 μg/kg). In particular the urine and milk were free of ochratoxin A and the metabolite. This finding contrasted with the findings of Ribelin, Fukushima & Still (1978), who detected significant amounts of ochratoxin A and ochratoxin α in the milk of one cow, but only following the administration of a large dose of the toxin.

Interstitial nephritis is common in adult cattle and although ochratoxin A is a potent nephrotoxin (Krogh *et al.* 1974), there seems to be no reason to implicate it in the case of these two cows. The possibility of an association is the subject of a current experiment, however. Apart from this possibility, ochratoxin A, like zearalenone, appears not to be 'carried-over' significantly into meat and milk when dietary concentrations of natural contaminants are in the 1–2 mg/kg range.

The accidental contamination of these experimental rations with aflatoxin B_1 produced some interesting results which raise important questions. The concentration of aflatoxin B_1 in the experimental rations was the maximum currently allowed in dairy rations in the UK by the Fertilisers and Feedingstuffs (Amendment) Regulations 1976 and it was to be expected, therefore, that no aflatoxin B_1 residues would be detected. Aflatoxin M_1 was detected in milk at approximately predictable levels however. That is, given that the ratio of aflatoxin B_1 in feed to aflatoxin M_1 in milk is 300 (Rodricks & Stoloff, 1977) the latter concentration would be about 0·07 μg/litre (cf. Table 1). The unexpected result was to find aflatoxin M_1 in the kidneys and urine of all four cows (Table 1). In particular, the mean concentration of aflatoxin M_1 in the kidneys (0·15 μg/kg) is 0·75% of the concentration of aflatoxin B_1 in the feed, which suggests that the 'carry-over' to bovine kidney is rather higher than to porcine kidney (Rodricks & Stoloff, 1977) and from the present data evidently higher than for bovine liver. It is possible, therefore, that the kidney is a sensitive indicator of aflatoxin exposure in cattle.

Although conclusions cannot safely be drawn from the present experiment in which so few animals were used, ochratoxin A may have influenced the accumulation of aflatoxin M_1 in the kidney; it will be seen that the mean level in this organ was at least twice as high in animals fed the ochratoxin-contaminated diet than in those exposed to zearalenone. Interactions between the common mycotoxins particularly at low dietary concentrations would appear to deserve further attention.

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Review Section

APPLICATION OF THE DIFFUSION THEORY TO MIGRATION OF PLASTICS COMPONENTS INTO PACKED GOODS: SURVEY OF RECENT MIGRATION STUDIES

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Summary—The transfer of ingredients of packaging materials into foods or a test food depends on the diffusion coefficient of the ingredient in the polymer (D_A^P) and the ratio of the concentrations of the ingredient in packaging material and food at equilibrium (determined by the Nernst partition coefficient). In type 1, the diffusion coefficient D_A^P of the additive A in the polymer P is nearly 0, and therefore little migration takes place apart from that resulting directly from a change in the interface-concentration of the additive by the contact with food (removing the additive from the surface of the packaging material). In type 2, the diffusion coefficient D_A^P is greater than zero and migration is measurable under the conditions and during the time of the intended contact (storage and distribution time). The diffusion coefficient in the packaging material is independent both of the kind of food and of time. In type 3, components of the food F can penetrate into the packaging material, causing swelling of the polymer and disturbing its physical structure. The consequence is normally an increase in the diffusion coefficient of the additive to D_A^{P+F} and consequently an increase in the migration rate. A system of general diffusion equations has been set up and applied on models to yield special mathematical equations, which permit the calculation of migration in the special cases mentioned above. The model criteria as well as application of the theory to special polymer/test food systems are presented.

Introduction

The interaction of food or cosmetics components with plastics packaging materials has become of increasing importance because of the legislation or other control procedures being developed for food and cosmetics packaging in the EEC (EEC Commission, 1978), USA (Food and Drug Administration, 1977), Federal Republic of Germany (Franck, 1977) and elsewhere. In order to protect the consumer, it is necessary to predict the migration, i.e. both the transfer of components of the packaging material into the foods or cosmetics and the transfer of components of the packaged material into the plastic. The latter transfer may result in changes in the properties of the plastics material. There have been and there will be a lot of investigations on the migration of these components, and it would be advantageous if such data could be used not only to establish the behaviour of the particular system under consideration but to predict the behaviour of other systems as well.

If the mechanism of migration can be understood well enough for mathematically exact descriptions to be possible, a few constants like the diffusion coefficient and the Nernst partition coefficient can be used to facilitate the description of migration behaviour of additives irrespective of the time of contact, the thickness of the samples or the concentrations used. It will probably be possible, as soon as sufficient data are available, to estimate the magnitude of the interaction of components from their chemical structure, thus obviating, in some cases, the need to determine the particular constants. Further, when the transfer mechanism has been exactly defined, the setting of legal limits for the composition of a packaging material (e.g. the maximum permitted concentration of each ingredient) will be as reliable as the limits on the concentration of such packaging ingredients in the food (levels which are normally very difficult to determine).

Mathematical models

General diffusion model

The basis for the transfer of ingredients from packaging materials into foods or test foods is the tendency of the ingredients to diffuse from areas of high concentration or, more correctly, of high chemical potential into areas of low chemical potential. This diffusion can only take place if the ingredient is sufficiently mobile, and for this mobility the diffusion coefficient D_A^P is characteristic. The diffusion proceeds more or less quickly until an overall constant chemical potential is reached, the resulting ratio of concentrations in the material and the food being determined by the Nernst partition coefficient.

According to experience with actual food–plastics material systems, three different types of system can be defined. These are similar to the types discussed by Knibbe (1971) and by Katan and his colleague (Briston & Katan, 1974; Katan, 1971).

In type 1, the diffusion coefficient D_A^P of the additive A in the polymer P is nearly 0, and therefore hardly any migration takes place except by a change in the interface concentration of the additive on contact with food (removing the additive from the surface of the packaging material).

In type 2, the diffusion coefficient D_A^P is definitely greater than zero and is such that the migration is measurable under the conditions and during the time of the intended contact (storage and distribution). D_A^P in the packaging material is independent both of the kind of food and of time.

In type 3, components of the food or test food (F) can penetrate into and interact with the packaging material, causing swelling of the polymer and disturbing its physical structure. The consequence is normally a larger diffusion coefficient D_A^{P+F} for a particular additive in the swollen layer and therefore the migration rate is increased.

Type 3 contains two sub-groups in which the interaction between the polymer and food increases D_A^P either from approximately 0 in the unswollen polymer to $D_A^{P+F} > 0$ in the swollen layer (sub-group *a*) or from > 0 in the unswollen polymer to $D_A^{P+F} > D_A^P$ in the swollen layer (sub-group *b*). In sub-group *a* of type 3, the diffusion coefficient D_A^P in the unswollen polymer is nearly zero, as in type 1, but it is increased by the swelling of the polymer at least to the type 2 level so that the diffusion becomes measurable. In sub-group *b* of type 3, the diffusion coefficient D_A^P is markedly greater than zero and is further increased by swelling.

F. B. Rudolph (unpublished work, 1978) has developed a theory that takes into consideration the simultaneous diffusion of the additive and the test food as well as the partition of the additive in the different phases. To apply these general equations to special systems, which have been investigated experimentally by vom Bruck, Eckert & Rudolph (1976), Figge (1972) and Figge & Koch (1973), a model had to be set up in order to determine boundary conditions for which the mathematical equations have been solved. With this, the theory has been proved using experimental data and the calculated diffusion parameters.

The model, schematically represented in Fig. 1, is

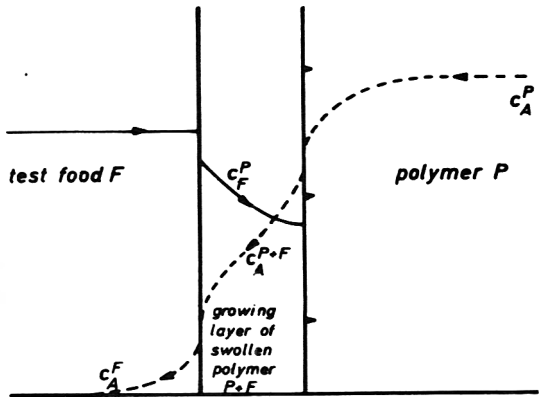

Fig. 1. Schematic representation of the distributions of the concentration C_F^P of food F and of the concentrations C_A^P, C_A^{P+F}, C_A^F of the additive in the different phases.

composed of three phases: the unswollen polymer P with an initially homogenous concentration of additive A, the food F, which is initially free from additive, and the swollen polymer layer P + F, the thickness of which is initially zero and increases with time. The polymer and the food are of infinite thickness. The diffusion coefficients of the food or food components in the polymer (D_F^P) and of the additive in the original polymer (D_A^P) and in the original food (D_A^F) are assumed to be independent of the concentrations. These approximations are possible because the concentration of additive is very low and the packaging material may not be penetrated totally by the food. The diffusion coefficient of the additive in the swollen polymer (D_A^{P+F}), however, may be an arbitrary function of the concentration of the food in the polymer, and there may be discontinuities in the concentration of the migrating additive and the migrating food at the interfaces of the phases because in the equilibrium the chemical potentials are identical in the different phases (Nernst).

These assumptions lead to the following mathematical solution of the diffusion equations (F. B. Rudolph, unpublished work, 1978):

$$M_A^F(t) = \frac{2C_A^P \cdot \sqrt{D_A^F}}{\Delta \cdot \sqrt{\pi}} \cdot \sqrt{t}$$

$$\Delta = \frac{\sqrt{D_A^F}}{\sqrt{D_A^P}} \cdot \exp\left[\frac{\eta_x^2}{D_A^P} - J(\eta_x^2)\right] \cdot \operatorname{erf}\left(\frac{\eta_x}{\sqrt{D_A^P}}\right) + \left\{\frac{2\sqrt{D_A^F}}{\sqrt{\pi}} \operatorname{Int}(\eta_x) + K_1\right\} \cdot \left\{K_2 - \frac{\sqrt{\pi}}{\sqrt{D_A^P}} \cdot \eta_x (K_2 - 1) \cdot \exp\left(\frac{\eta_x^2}{D_A^P}\right) \cdot \operatorname{erfc}\left(\frac{\eta_x}{\sqrt{D_A^P}}\right)\right\}$$

$$\operatorname{Int}(\eta) = \int_0^{\eta} \frac{\exp(-J(u^2))}{D_A^{P+F}(u)}\, du \qquad \text{and} \qquad J(\eta^2) = \int_0^{\eta} \frac{du^2}{D_A^{P+F}(u)}$$

where

$M_A^F(t)$ = amount of additive in food,
C_A^P = initial concentration of additive in polymer,
D_A^F = diffusion coefficient of additive in food,
D_A^P = diffusion coefficient of additive in polymer,
D_A^{P+F} = diffusion coefficient of additive in swollen polymer layer,
$\frac{\eta_x}{\sqrt{t}}$ = velocity of propagation of interface between swollen polymer and original polymer,
K_1 and K_2 are partition coefficients at the interfaces.

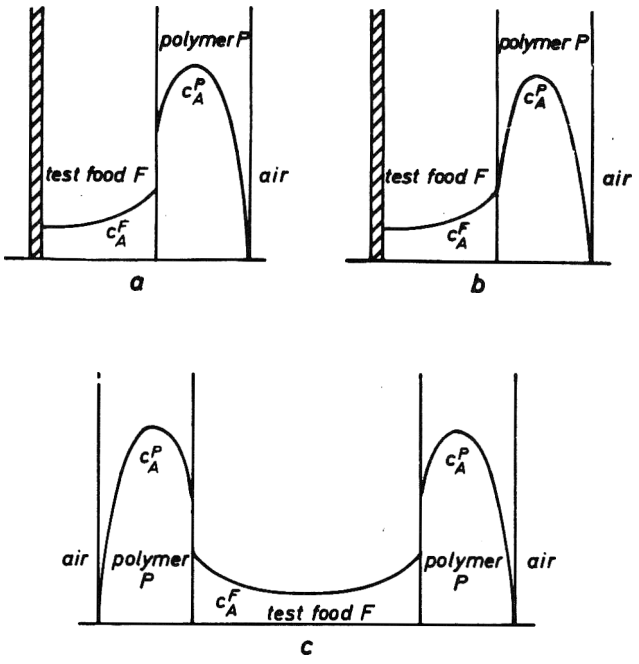

Fig. 2. Schematic representation of the distribution of an additive in the polymer–food systems under consideration.

Special model for volatile additives

For volatile additives like vinyl chloride in a system of three phases, we use a solution of the second Fick equation. For such additives it is important to take layers of food and polymers of finite thickness into consideration. The polymer is interfaced by air and by the food. The food is interfaced by the polymer and by an impermeable 'wall' (Fig. 2a,b) or in a symmetrical food–polymer–air system (Fig. 2c) by polymer layers on both sides. The systems represented in Figs 2a and 2b differ only in respect of a concentration discontinuity at the interface of polymer and food. The diffusion coefficients of additive in food and in polymer are assumed to be constant.

The solutions of the model equations for volatile additives are:

(i) when the food is stirred ($D_A^F \rightarrow \infty$; Fig. 3, curve a)

$$M_A^F(t) = 2C_A^P \sum_{n=0}^{\infty} \frac{\{1 - \cos[\mu_n \cdot (l_F - l_P)]\}\, e^{-D_A^P \mu_n^2 t}}{\mu_n \{l_F \sin[\mu_n (l_F - l_P)] + \mu_n l_P (l_F - l_P) \cos[\mu_n (l_F - l_P)]\}}$$

where the μ_n values are the roots of the transcendental equation

$$\operatorname{tg}[\mu_n (l_F - l_P)] = \frac{1}{l_P \cdot \mu_n}$$

and l_F and l_P are the thickness of the food and the polymer layer.

(ii) where the diffusion coefficients of the additive in the food and in the polymer are equal ($D_A^P = D_A^F = D$; Fig. 3, curve b)

$$M_A^F(t) = -\frac{8C_A^P}{\pi^2} \frac{l_F}{l_P} \cdot \sum_{n=1}^{\infty} \frac{1}{(2n-1)^2} \cdot \sin\left[\frac{(2n-1)}{2} \frac{l_P}{l_F}\right] \cdot \left\{(-1)^n + \sin\left[\frac{(2n-1)\pi}{2} \frac{l_P}{l_F}\right]\right\} \cdot \exp\left(\frac{-D}{l_F^2} \frac{(2n-1)^2 \pi^2}{4} t\right)$$

For volatile additives like the monomers, an even distribution in the polymer can only be expected immediately after the processing of the package (vom Bruck *et al.* 1976); after some storage the vinyl chloride distribution within the PVC will depend on the temperature and time of storage in air. We have calculated a curve (Fig. 3, curve c) for a PVC, both sides of which were in contact with air before the contact with food. The measured values fit this theoretical curve c well.

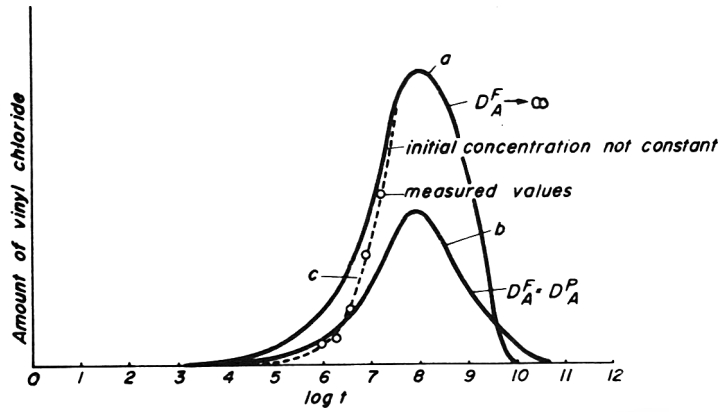

Fig. 3. Amount of vinyl chloride [$M_V^F(t)$] in HB 307 as a function of time (at 20 C and with one-sided contact between the PVC and HB 307).

Diffusion with simultaneous sorption

In addition, we can consider a system where the additives under consideration are not all free but are bound in part to higher energy sites in the polymer. In this case, the concentration of the free molecules of the additive can be calculated from the analytical or total concentration depending on the type of polymer–additive interaction. The desorption kinetics can be described by a thermodynamic *or* a statistical method, such as the Langmuir sorption mechanism (Berens, 1975; Gilbert, 1977).

Comparison of theoretical curves and experimental data

It is of interest to consider some actual systems in relation to these various models. Figure 4 (curve a) shows the behaviour of a system comprising a tin stabilizer in PVC in contact with sunflower oil. The experimental migration data are taken from the literature (Figge, 1972; Figge & Koch, 1973). This system represents type 1 of the general diffusion model. By the contact with sunflower oil the interfacial tension of the polymer may be changed, and therefore the interface concentration of the tin stabilizer will change. The surplus will diffuse into the oil, but only from the surface and not from within the PVC.

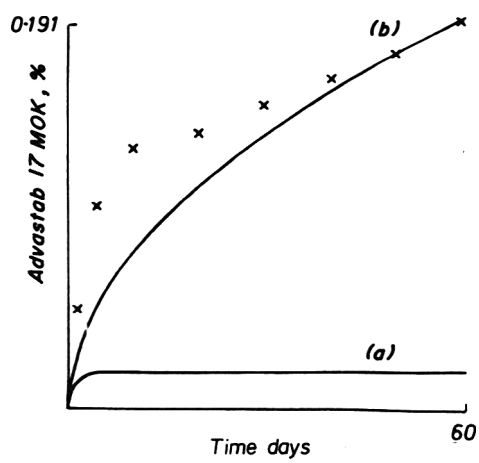

Fig. 4. Diffusion of the organotin stabilizer Advastab 17 MOK from PVC into (a) sunflowerseed oil and (b) tricaprylin.

Also in Fig. 4, the migration of the tin stabilizer into tricaprylin has been plotted (curve b). In this system a definite swelling of the PVC must be expected even at 20 C; at 65 C the PVC is slowly dissolved. This case represents an example of the type 3a system of the general diffusion model as the tin stabilizer does not migrate out of the undisturbed PVC but migrates in measurable amounts out of the swollen PVC. The experimental data (Figge, 1972; Figge & Koch, 1973) fit the curve quite well at the later stage, whereas at the beginning the migration rate is higher than the calculated one, a situation that may be due to an interfacial effect similar to that discussed for curve 4a.

Figge, Bieber & Rudolph (1979) have assembled some data (Fig. 5) on a phenolic antioxidant in polypropylene, a system of type 3b, and there is an excellent fit between our calculated values and the migration values determined experimentally. The difference between the migration into aqueous acetic acid, which does not swell the polypropylene, and that into test fat HB 307 (Figge, Eder & Piater, 1972), which does swell this polymer, is obvious.

An example (vom Bruck *et al.* 1976) for type 2, a readily measurable diffusion not disturbed by the swelling of the polymer by the food components, is the migration of vinyl chloride out of PVC into the test fat HB 307. The experimental points (Fig. 3) fit well with the calculated curve, which takes into account, as already mentioned, that we had a vinyl chloride distribution in the PVC containers before the contact with HB 307, as these had been stored for some time in the open air before filling.

A further example for type 3b is the system acrylonitrile in ABS (Fig. 6), in which there is a definite increase in the migration of acrylonitrile into water compared with migration into test fat HB 307. Here again, the theoretical curves fit the experimental data well. This last example shows that it is always a proof of an interaction (i.e. a swelling of the polymer by

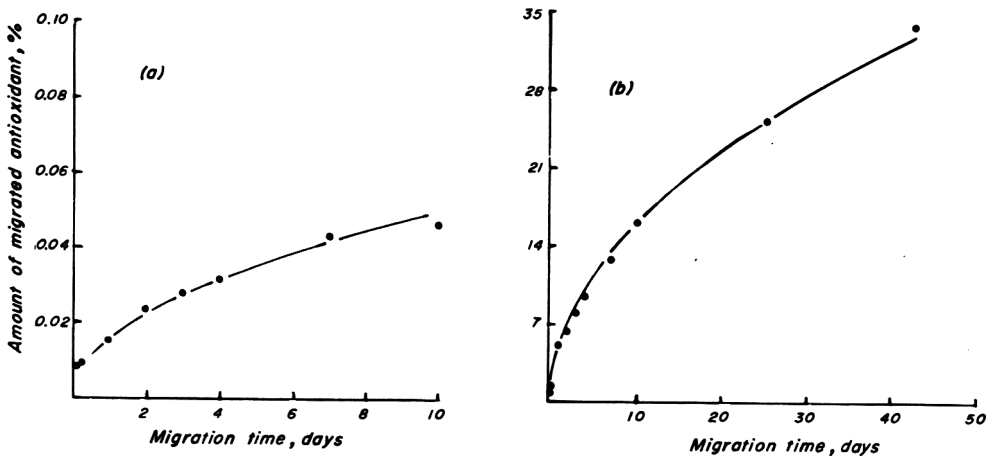

Fig. 5. Time dependence of the migration of antioxidant from polypropylene into two different food simulants, (a) 3% (w/w) acetic acid and (b) test fat HB 307: ●, experimental values; ——, model curve.

at least one food component) if, under identical migration conditions with a different food, much higher migration rates are found. It should be established, however, that such a difference is not due to a great difference in the Nernst distribution coefficient.

Conclusions

Thus we are able to calculate the diffusion parameters from experimental migration data. *Vice versa* we shall be able to calculate the specific migration of particular additives into foods as soon as the appropriate test food has been identified and the above-mentioned diffusion parameters in the intended polymer have been determined at the temperature of use. The influence of temperature on the diffusion will be predictable in areas where there are no structural changes in the polymer, e.g. those distant enough from the glass transition area.

Because of this clear relationship, limits on packaging components can be as reliable as limits on levels of food contamination. This should be considered in discussions relating to draft directives on food-packaging materials.

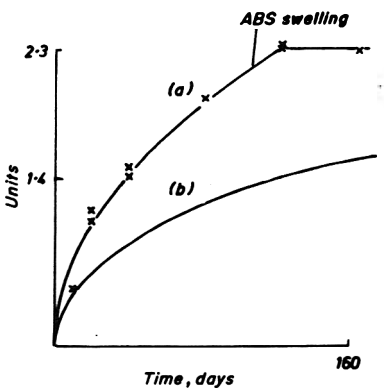

Fig. 6. Diffusion of acrylonitrile from ABS into (a) water and (b) test fat.

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CANCER, MATHEMATICAL MODELS AND AFLATOXIN

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Summary—When cancer is caused in laboratory animals by a socially-valuable chemical or an unavoidable environmental contaminant, natural or otherwise, the risks to man from exposure to the chemical at very much lower doses must be estimated. The choice of a mathematical model is an important step in this assessment. In this paper, several available models are evaluated with respect to aflatoxin, one of the few agents with usable results both from experiments with animals and from surveys in man.

Introduction

A pressing problem for our society is the interpretation of results from lifetime feeding studies that produce cancer in laboratory animals.

In this paper, one aspect of the problem is explored statistically in detail for a particular agent, aflatoxin. The aspect selected is the choice of a mathematical model (function) to be fitted to the observed results from the animal experiment and used to extrapolate these results to much lower dose levels and to estimate the risk to man. Several models have been proposed for this step.

Such a mathematical model could be evaluated by applying it to the results from animal experiments with several agents and checking the predicted risks against observed human risks demonstrated in epidemiological studies of the same agents. Unfortunately, appropriate human epidemiological results are available for very few agents, amongst which aflatoxin is the most promising.

To discriminate between the models it is valuable to use an animal experiment with several dose levels showing a clear dose–response relationship. One such experimment using five doses of aflatoxin (but only male rats) has recently appeared, and is the main study included below. Four other experiments in male rats with fewer dose levels are considered.

Epidemiological studies

Epidemiological studies of liver cancer and aflatoxin ingestion in man have been conducted in Asia and Africa. Although there were variations among the study designs, a general pattern does emerge. A region with a high incidence of deaths from liver cancer was chosen for study, and other regions of the same country were usually included for comparison. The investigators confined their studies to rural areas believing that these regions have been stable for many years with respect to the composition of the population and of the diet.

It was necessary to estimate the incidence of liver cancer among the population by confirming the causes of individual deaths as far as possible and to estimate aflatoxin ingestion among the population. A carefully designed plan was followed to sample the meals consumed by the residents. In every study, the investigators concluded that there was a positive correlation between the incidence of liver cancer and the level of ingestion of aflatoxin. They were reluctant to declare a cause-and-effect relation because of the possibility of some unknown factor correlated with aflatoxin ingestion (such as malnutrition). They also pointed out that the foods containing aflatoxin are extremely important to the populations at risk.

Table 1 gives the reported data and the results of some calculations. Considering the Songkhla region in Thailand, the estimated total male population is 48,934, and the male life expectancy from birth is 54 yr (United Nations, 1975). Deaths from liver cancer were monitored for a year, and two were observed. The corresponding yearly death risk is $4{\cdot}1 \times 10^{-5}$ ($=2/48{,}934$) and the corresponding lifetime risk is 221×10^{-5} ($=4{\cdot}1 \times 10^{-5} \times 54$). The survey of diets lasted one year, and the total aflatoxin (all types, but mostly B_1) was measured. A total of 922 meals were analysed in the 1-yr diet survey yielding a mean aflatoxin dose of 6·5 ng/kg body weight/day.

The mean aflatoxin dose reported in Table 1 requires some comment. In all regions, the great majority of meals had no measurable aflatoxin content. Meals with positive contents showed huge daily and annual variations as well as big differences from person-to-person within the region. By contrast, animal studies in laboratories have essentially constant daily ingestions over the lifetimes of the animals. Fig. 1 shows the dose–response relation between aflatoxin dose and lifetime risk of death from liver cancer in man using the data for the first nine regions in Table 1. For each point, there are considerable uncertainties about both the dose and the death rate. The 99% confidence intervals (Poisson approximation) for the death rates are indicated.

It is important to estimate the background liver-cancer risk corresponding to no ingestion of aflatoxin in a region. A straight line can be fitted to the data in Fig. 1, and the intercept of this straight line then represents the background rate. The dietary intake

Table 1. *Data from epidemiological studies in man (males only)*

Region	Total male population at risk	Male life expectancy (yr)	No. of cases of liver cancer in males during no. of years in brackets	Yearly risk ($\times 10^5$)	Lifetime risk ($\times 10^5$)	Duration of diet study (yr)	Type of aflatoxin	Total no. of meals analysed	Mean dose of aflatoxin (ng/kg/day)	Observed lifetime risk from aflatoxin ($\times 10^4$)
THAILAND										
Songkhla*	48934†	54	2(1)	4·1	221	1	total	922	6·5	100
Ratburi*	49768†	54	5 (1)	10·0	542	1	total	1005	61·0	421
KENYA										
High region‡	18394	47	1 (4)	1·4	64	1·75	B	808	4·17	0
Middle‡	75138	47	13 (4)	4·3	203	1·75	B	808	6·85	82
Low‡	68808	47	16 (4)	5·8	273	1·75	B	816	12·42	152
SWAZILAND										
Highveld§	48628	41	9 (5)	3·7	152	1	?	288	8·34	31
Middleveld§	69136	41	24 (5)	6·9	285	1	?	288	14·43	164
Lowveld§	45814	41	35 (5)	15·3	626	1	?	288	53·34	505
Lebombo§	8713	41	4 (5)	9·2	376	1	?	192	19·89	255
MOZAMBIQUE										
Inhambane‖		41	?(3)	35·0	1435	?	total	880	222·4	1314

*Shank, Bhamaropravati, Gordon & Wogan, 1972; Shank, Gordon, Wogan, Nondasuta & Subhamana, 1972.
†Indicates one-half of the total population.
‡Peers & Linsell, 1973.
§Peers, Gilman & Linsell, 1976.
‖Van Rensberg, Van der Watt, Purchase, Pereira Coutinho & Markham, 1974.

Table 2. *Liver tumour data from aflatoxin feeding studies with rats (data from males only)*

Study no.	Strain of rats	Type of aflatoxin	Incidence of liver cancer at aflatoxin doses (ppb) of								
			0	1	5	10	15	20	50	100	500
I*	Fischer	Crystalline B_1	0/18 (0)	2/22(0·09)	1/22 (0·05)	—	4/21 (0·19)	—	20/25 (0·80)	28/28 (1·0)	—
II†	Fischer	Spectrally pure B_1	0/16 (0)	—	—	—	—	5/13 (0·38)	—	—	—
III†	Wistar	Spectrally pure B_1	0/17 (0)	—	—	—	—	0/20 (0)	—	7/17 (0.41)	—
IV‡	Porton	Rosetti (98% B_1)	0/46 (0)	—	—	—	—	—	—	17/26 (0·47)	25/25 (1·0)
V§	USC	B_1 from peanuts	0/9 (0)	0/16 (0)	—	0/10 (0)	—	—	—	—	—

*Wogan, Paglialunga & Newberne (1974).
†Nixon, Sinnhuber, Lee, Landers & Harr (1974).
‡Butler & Barnes (1968).
§Alfin–Slater, Aftergood, Hernandex, Stern & Malnick (1969).

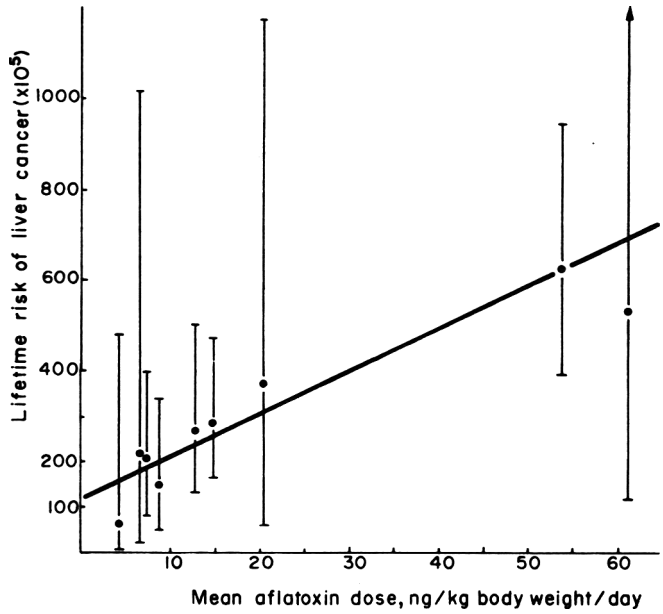

Fig. 1. The dose–response relationship between aflatoxin intake and lifetime risk of liver cancer in man, from epidemiological data.

(horizontal axis) was assumed to have been measured without error. An iterated, weighted least-squares procedure was used to account for the error in the observed cancer risks (vertical axis). Statistically, the fit is very good in that the residuals shown in Fig. 1 are easily explained by chance. The estimated function is: lifetime risk $= \alpha + \beta$ dose (in ng/kg body weight/day), where α is estimated as 0·00121 with a standard error of 0·00039, and β is estimated as 0·000096 with a standard error of 0·000022, and α is the background risk.

It is now necessary to assume a cause-effect relationship between aflatoxin ingestion and human liver cancer. The difference between the observed life-time risk in a region and the background lifetime risk just estimated (0·00121) is then the observed lifetime risk caused by the ingestion of aflatoxin in that region (See Table 1).

The results for the Inhambane region of Mozambique were not used in the above analysis. The ingestion in Inhambane (222·4) is much higher than in any other region, and it was feared that this extreme data point would have a disproportionate effect on the estimated background risk. In fact, the data from Inhambane are generally incomplete and were not used in what follows.

There are a number of problems involved in applying this regression model to man:

Estimated lifetime risk from aflatoxin = 0·000096 × (mean intake in ng/kg/day).

For example, assume a region (society) with a mean dose of 1 ng/kg/day. This corresponds to an estimated aflatoxin lifetime risk of 0·000096 ($9{\cdot}6 \times 10^{-5}$) for that region as a whole, but this risk does not necessarily apply to any individual. It is also conceivable that there is a threshold above 1 ng/kg for male humans and that individuals ingesting aflatoxin at this level experience zero aflatoxin risk. This difficulty arises here because all the humans in a region did not ingest aflatoxin at the average rate. Nevertheless the means for each study result in a straight line (Fig. 1).

Experimental studies in the rat

The experimental results from five lifetime (24 months) feeding studies with male rats are given in Table 2. The endpoint is liver cancer (not including precancerous lesions). Study I is of primary interest here. Fischer rats were fed crystalline aflatoxin B_1 at dose levels of 0, 1, 5, 15, 50 and 100 ppb*.

Study II also used Fischer rats and the result is consistent with the results from study I. Studies III, IV and V used other strains, which seem to be less sensitive to aflatoxin, but the results illustrate some of the usual problems in low-risk extrapolation. Study III has two doses, but one with a completely negative response while study IV with two doses, has one completely positive response. Study V has completely negative results; taken alone it shows no evidence of the carcinogenicity of aflatoxin.

Mathematical models

Each of four models was fitted to the animal results in Table 2 (especially study I) and the resulting calculated risks for man ingesting low levels of aflatoxin (column 10 of Table 1) were compared with the observed aflatoxin risks in man (column 11 of Table 1). The four models are the probit, logit, one-hit and improved Mantel-Bryan procedure (Mantel, Bohidar, Brown, Ciminera & Tukey, 1975; Mantel-Bryan, 1961). The mathematical forms of the first three

*$b = 10^9$

models are as follows:

(i) *Probit model.* $P = F(Y)$ where F is the unit normal distribution function and $E(Y) = \alpha + \beta \log_{10} x - 5$

(ii) *Logit model.* $P = 1/(1 + e^{-Y})$ where $E(Y) = \alpha + \beta \log_{10} x$

(iii) *One-hit model.* $P = 1 - e^{-\beta x}$

In each model, P is the probability of an animal's developing liver cancer when fed at the dose level of x, Y is the probit or logit, respectively and α and β are parameters to be estimated from the data.

(iv) *Mantel-Bryan model.* This is essentially a probit model with the slope (β) arbitrarily set at a 'conservative' level of 1·0 and the intercept (α) estimated from the data.

For the probit, logit or one-hit model, two estimates of risk are considered here: a best estimate in the statistical sense, and an upper 99% confidence limit. In its intended use, the Mantel-Bryan model provides only an upper confidence limit to the risk at low doses.

Other models are frequently discussed. Two of them may be thought of as generalizations of the one-hit model to be applied when that model does not fit the data. The first is the k-hit model. Mathematically, it is a gamma-distribution function with a second parameter k. When $k = 1$, it reduces to the one-hit model. The second (Hartley & Sielken, 1977) generalizes the one-hit model in another direction:

$$P = 1 - e^{-\beta_1 x - \beta_2 x^2 - \ldots - \beta_m x^m},$$

where m is determined from the data. It too reduces to the one-hit model when $m = 1$. Neither of these models will be applied to the data in Table 1, mainly because the one-hit model does fit the results from study I.

Another model recently suggested (Cornfield, 1977) attempts to follow a biological theory of carcinogenesis rather than a formal mathematical course. Since the statistical procedures required to fit this model to data are not at present available, it will not be used here.

Still other models use the time-to-tumour approach (Hartley & Sielken, 1977; Hoel, Gaylor, Kirschstein, Saffiotti & Schneiderman, 1975). Their advantage is that they estimate the life-shortening effect as well as the overall risk. Since the necessary time-to-tumour information in the rat experiments is not available, these models will not be used here.

Consider study I in Table 2 first. Table 3 gives the maximum-likelihood estimates of the relevant parameters for each of the four models of interest (for the dose x in ppb). Table 4 gives the calculated frequencies which follow from these estimates. The results are typical. All the models fit the data about equally well. There is no clear best fit. There are sufficient data here to show that the calculated frequencies for the one-hit model match the observed frequencies within the limits of statistical error ($P = 0{\cdot}17$, observed chi-square = 3·48 with 2 d.f., 1 and 5 ppb groups pooled, 50 and 100 ppb groups pooled). There are insufficient data to carry out this test for the probit or logit models (necessary pooling produces zero degrees of freedom).

Table 3. *Parameters for study I estimated using the various mathematical models*

Mathematical model	Parameter	
	α	β
Probit	2·537	1·906
Logit	−5·075	3·785
One-hit	—	0·03013
Mantel-Bryan (upper 0·99)	−1·499	—

Extrapolations to human epidemiological observations

With any estimated model in Table 3, it is now possible to calculate the risk (extrapolated) at any low dose (in ppb) for the male rat. The dose in ppb can then be converted to its equivalent in ng/kg body weight/day, where 1 ppb equals 50 ng/kg/day for the adult rat. Assuming body-weight conversion between the rat and man, this gives the risk–dose relationship for man. Figure 2 shows the picture for the probit (P), logit (L) and one-hit (H) models applied to study I. Logarithmic scales are used only for convenience. Figure 3 with dashed curves shows the picture for the corresponding upper 99% confidence limits. Here, the Mantel-Bryan model (M) is included. It is possible to fit the one-hit model to each of the other four studies in Table 2. Figure 4 shows the upper 99% confidence-limit extrapolation for each study separately. It is also possible to apply the Mantel-Bryan model to each of the other four studies in Table 2. Figure 5 shows the picture.

Finally, each of the first three models (P, L and H) can be applied to all the data in Table 2 for all the studies. In no case is the fit statistically satisfying, or close to it, because the Fischer strain is different from the others. Nevertheless, these 'best' fitting functions give crude summaries of all the studies with rats, and their graphs are shown as solid curves in Fig. 6. The dashed curve is the average of the five curves in Fig. 5.

This is an appropriate place to mention three other aspects of the overall problem, which are not related to the choice of a model. The first is species conversion. One suggested alternative to body-weight conversion is the concentration in the diet. The effect of this when converting from the rat to man is to raise the extrapolated risk for man by a factor of about two; that is, the curves in Figs 2–6 would be higher by this factor. The second aspect is the choice of an end-point for the animal studies. One suggested

Table 4. *Calculated frequencies of liver cancer predicted by the three models from the data of study I*

Dose (ppb)	No. at risk	Observed frequency	Probit frequency	Logit frequency	One-hit frequency
1	22	2	0·1	0·1	0·6
5	22	1	2·8	1·8	3·1
15	21	4	8·7	7·3	7·6
50	25	20	19·5	19·9	19·5
100	28	28	25·5	25·9	26·6

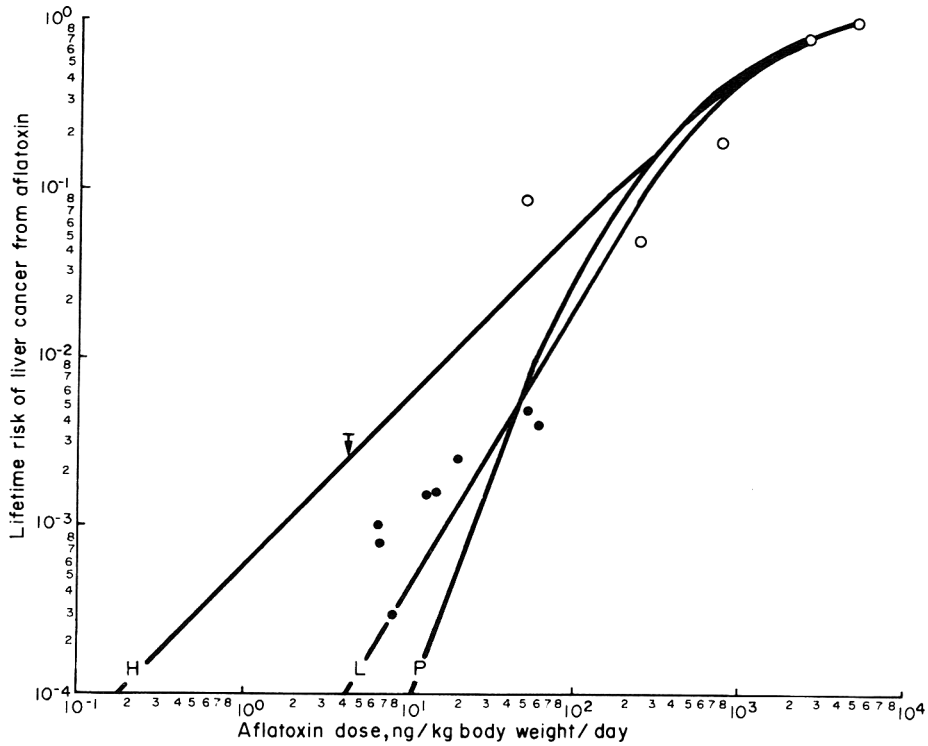

Fig. 2. Best estimates of the relationship between aflatoxin dose and lifetime risk of liver cancer in rats, estimated from the probit (P), logit (L) and one-hit (H) models, using the data of Wogan *et al.* (1974; study I). Observed data for rats (○); observed aflatoxin lifetime risk for man (●); upper 99% confidence limit for an observed human risk of zero (↓).

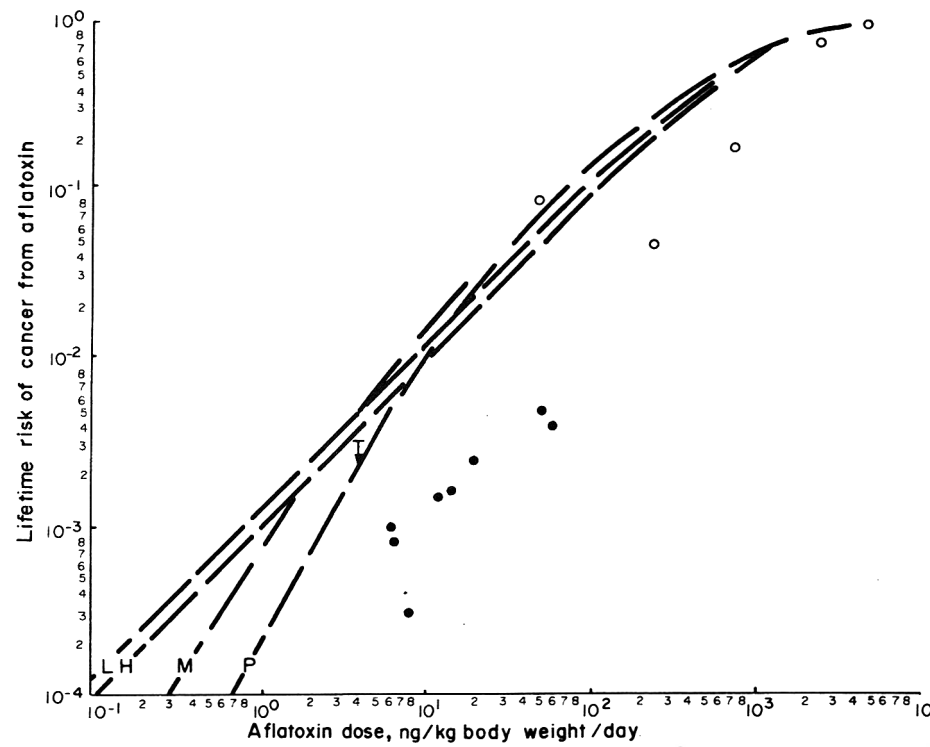

Fig. 3. Upper 99% confidence limits from the probit (P), logit (L), one-hit (H) and Mantel-Bryan (M) models for the dose–response relationship between aflatoxin intake and risk of liver cancer in the rat using the data of Wogan *et al.* Observed data for rats (○); observed aflatoxin lifetime risks for man (●); upper 99% confidence limit for an observed human risk of zero (↓).

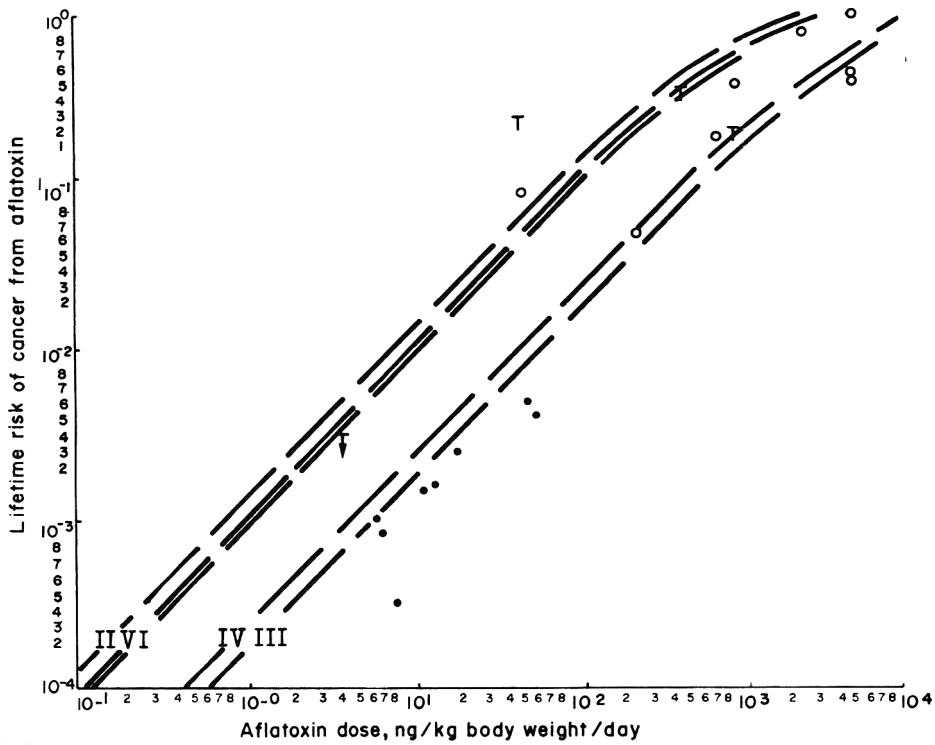

Fig. 4. Upper 99% confidence-limits from the one-hit model for the dose-response relationship between aflatoxin intake and risk of liver cancer in the rat using the data of each study separately. Observed data for rats (O): observed aflatoxin lifetime risks for man (●): upper 99% confidence limit for an observed human risk of zero (↓): upper 99% confidence limit for an observed risk of zero for a group of rats (T).

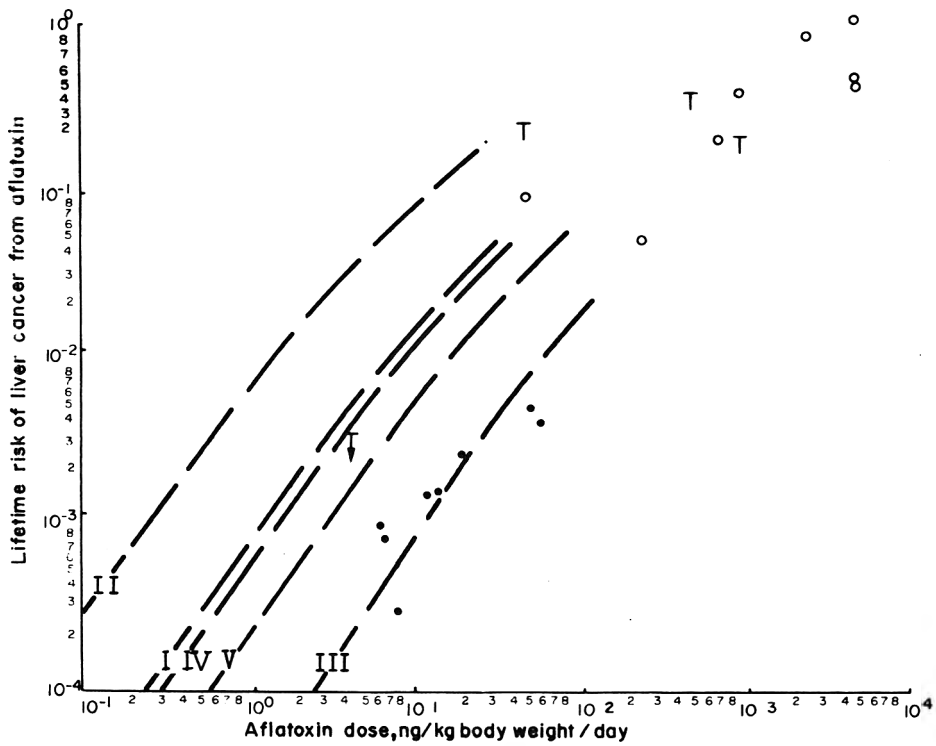

Fig. 5. Upper 99% confidence limit extrapolation from the Mantel-Bryan model for the dose-response relationship between aflatoxin intake and risk of liver cancer in the rat using the data of each study separately. Observed data for rats (O): observed aflatoxin lifetime risk for man (●): upper 99% confidence limit for an observed human risk of zero (↓). Upper 99% confidence limit for an observed risk of zero for a group of rats (T).

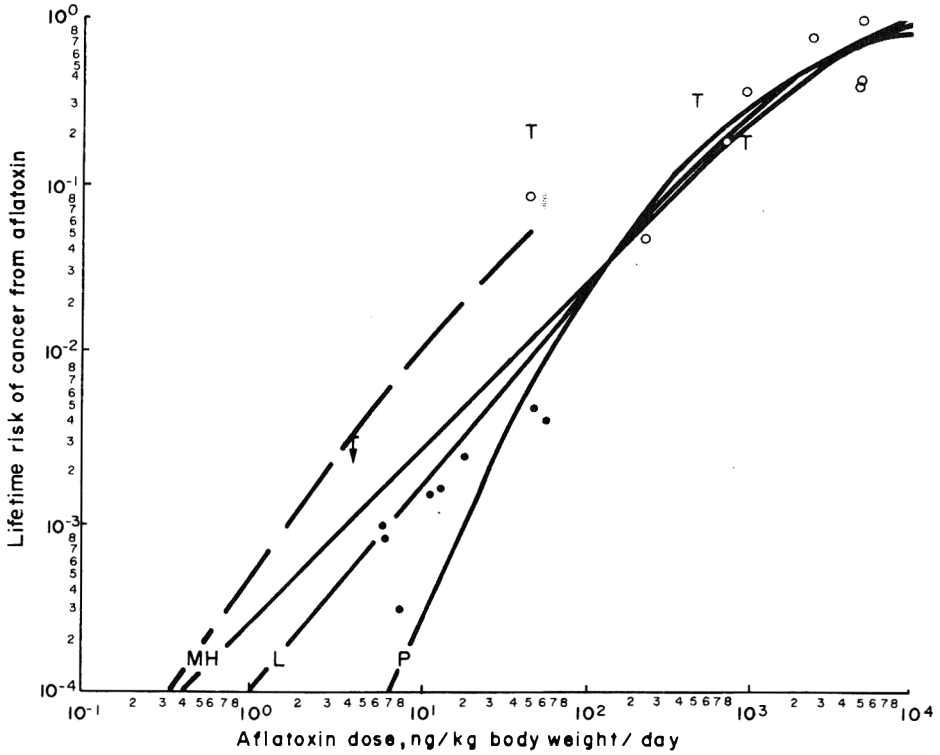

Fig. 6. Best estimates of the dose–response relationship between aflatoxin intake and risk of liver cancer in the rat using each of the models for all of the rat data combined: probit (P), logit (L), one-hit (H) and Mantel-Bryan (M). Observed data for rats (O); observed aflatoxin lifetime risks for man (●); upper 99% confidence limits for an observed human risk of zero (↓); upper 99% confidence limit for an observed risk of zero for a group of rats (⊤).

alternative is cancer or a pre-cancerous lesion. For the one-hit and the Mantel-Bryan models, this has the general effect of raising the curves; for the other two models the effect is less clear. The third aspect is species selection. With aflatoxin, there is a great range: the mouse does not seem to develop liver cancer; the duckling appears to be much more sensitive than the rat.

It is premature to draw conclusions, and none will be attempted. The hope is that these data can be used for other models as they are suggested and that other agents can be studied along these lines. Our society, through its regulators, makes frequent risk calculations on the basis of results from animal studies. The person making such a calculation must make many choices (model, confidence levels, species conversion, end-point, species selection and so on) to reach his final estimate. The effects of all these choices on the final estimate are huge (even larger than indicated in the next section). One way to narrow the range is to study agents like aflatoxin for which there are both animal and human data. Even studies in man showing no effect, for example, might be valuable to reject over-conservative procedures of risk calculation.

Virtual Safe Dose

With any of the models discussed here, it is impossible for an animal experiment with an agent to produce an extrapolated lifetime risk of zero at any dose, however small. What is an acceptable lifetime risk? One answer can be obtained by a risk-benefit calculation. Another answer is through the choice of some arbitrary small risk. The FDA (1977) in a similar situation with residues in meat has chosen one in a million (10^{-6}) as an acceptable lifetime risk. For the entire US population, a 10^{-6} lifetime risk is equivalent to about three cases of cancer per year from the agent. Actually, the FDA procedure uses the 10^{-6} risk as an upper limit, rather than a best estimate.

With each of the estimated models above, one can calculate the daily dose of aflatoxin for human males corresponding to an overall 10^{-6} lifetime risk of cancer, or 1·5 cases/year among US males. Figures 2–6 are of no use for this because 10^{-4} is the lowest plotted level. Table 5 gives the calculated results. There is a great range in the concentration in solid food ($0{\cdot}029 \times 10^{-3}$ to 0·11 ppb) expected to produce a 1 in 10^6 risk of liver cancer.

Aflatoxin ingestion in the US comes mainly from peanuts (Stoloff, 1976). The concentration of aflatoxin in consumer peanuts has been roughly estimated as at least 1·5 ppb in 1975, and more in the two preceding years (Stoloff, 1976; Table 4). In recent years, the average consumption of peanuts in the US has been about 6 pounds (2·7 kg) per person (US Bureau of Census, 1977). For a person eating 2 kg of moist solid food per day, these estimates imply an average concentration of at least 0·011 ppb in the US diet: $(2{\cdot}7 \times 1{\cdot}5)/(365 \times 2) = 0{\cdot}011$ ppb. Of course, we have

Table 5 *Virtual safe doses and expected numbers of cases of liver cancer among US males, calculated using the various models*

Mathematical model used	Data from studies in man	Data from rat study I only		Data from all five rat studies	
	Best estimate	Best estimate	99% confidence limit	Best estimate	99% confidence limit
	Calculated intake (ppb) resulting in a $1/10^6$ risk of liver cancer*				
Probit	—	0·11	0·0037	0·054	—
Logit	—	0·0086	$0·029 \times 10^{-3}$	$0·77 \times 10^{-3}$	—
One-hit	$0·36 \times 10^{-3}$	$0·058 \times 10^{-3}$	$0·039 \times 10^{-3}$	$0·14 \times 10^{-3}$	—
Mantel-Bryan	—	—	$0·98 \times 10^{-3}$	—	$1·3 \times 10^{-3}$
	Calculated no. of cancer cases in US males resulting from an intake of 0·011 ppb aflatoxin†				
Probit	—	0·01	22	0·01	—
Logit	—	2	540	36	—
One-hit	46	290	430	120	—
Mantel-Bryan	—	—	160	—	96

*This is a lifetime risk (assuming that a dietary level of 0·035 ppb is equivalent to a daily intake of 1 ng/kg body weight/day for a 70-kg person ingesting 2 kg of moist solid food per day).
†The estimated average concentration of aflatoxin in the US diet is 0·011 ppb.

no way of knowing the concentration in peanuts and other foods prior to 1960, when aflatoxin was discovered.

Finally, this estimated concentration for the US population can be used with each model to calculate the corresponding yearly number of liver cancer cases attributable to aflatoxin among US males. These results are given in Table 5. For comparison, there are about 3500 liver cancers each year among US males.

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REVIEWS OF RECENT PUBLICATIONS

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Some *N*-Nitroso Compounds. Vol. 17. International Agency for Research on Cancer, Lyon, 1978. pp. 365. Sw.fr. 50.00 (available in the UK through HMSO).

The seventeenth volume of these IARC Monographs evaluates the carcinogenic risk associated with 18 *N*-nitroso compounds. The nitrosamines, selected on the grounds of their environmental occurrence and known profile of cancer testing, are *N*-nitroso-*n*-butylamine, *N*-nitrosodiethanolamine, *N*-nitrosodiethylamine, *N*-nitrosodimethylamine, *N*-nitrosodi-*n*-propylamine, *N*-nitrosofolic acid, *N*-nitrosomethylethylamine, *N*-nitrosomethylvinylamine, *N*-nitrosomorpholine, *N'*-nitrosonornicotine, *N*-nitrosopiperidine, *N*-nitrosoproline, *N*-nitrosohydroxyproline, *N*-nitrosopyrrolidine and *N*-nitrososarcosine. Several of these were the subject of earlier monographs, now revised and extended. Also considered are the nitrosamides, *N*-nitroso-*N*-ethylurea and *N*-nitroso-*N*-methylurea, together with the structurally-related anticancer drug streptozotocin. As is customary in this series, there is an opening section on principles for evaluating the cancer risk of chemicals. This is followed by a chapter on the carcinogenic status of nitrosamines, preceding the individual monographs. A cumulative index provides a rapid means of locating any of the several hundred monographs completed to date; location of work by author is restricted to an alphabetical list of references specific to each monograph.

Various agencies in at least 24 countries are known to consult the IARC Monographs on a routine basis, more perhaps for reliable summaries of carcinogenicity data than for any bold estimates of human hazard. A perusal through the first 17 volumes, rather than merely satiating the enquiring or encyclopaedic mind, will be a reassuring exercise for those who are required to use animal data in extrapolations to possible human risk. As is pointed out in the 'preamble' to volume 17, about 26 of the chemicals or manufacturing processes covered so far in this series are now generally accepted to cause cancer in man, and in this group all but possibly two of those that have been tested appropriately have produced cancer in at least one animal species, the possible exceptions being arsenic and benzene. For several of the group (aflatoxin, 4-aminobiphenyl, diethylstilboestrol, melphalan, mustard gas and vinyl chloride), the demonstration of carcinogenicity in animals preceded the evidence obtained from epidemiological studies or case reports. Such considerations appear to have provoked some change in the attitude of IARC experts towards the use of animal data. Data that provide "sufficient" evidence of carcinogenicity (i.e. evidence of an increase in malignant tumours) are now regarded as providing a reasonable basis for treating a compound in practical terms as if it were a human carcinogen. Only three of the compounds considered in volume 17 have "insufficient" incriminating evidence from animal studies. The lack of useful data (from any source) prohibited any evaluation of *N*-nitrosofolic acid, *N*-nitrosoproline and *N*-nitrosohydroxyproline.

It is noteworthy that the results of short-term positive tests are being incorporated to an increasing extent into IARC Monographs. The Working Group considers positive results in validated short-term tests as an indication of potential carcinogenicity, but stresses that negative results cannot be considered sufficient evidence to rule out the possibility of such a response.

In all, the latest monographs in the IARC series must be strongly recommended, particularly to those who have neither the time nor perhaps the inclination to examine the primary literature on nitrosamine carcinogenicity.

Lead Pollution in Birmingham. A Report of the Joint Working Party on Lead Pollution around Gravelly Hill. Pollution Paper No. 14. Central Unit on Environmental Pollution; Department of the Environment. HMSO, London, 1978. pp. viii + 110. £3.75.

Public concern over the effect of lead pollution has centred around the Gravelly Hill M6–A38 motorway interchange (Spaghetti Junction) for a number of years. In 1974 a Joint Working Party on Lead Pollution around Gravelly Hill was set up by the Department of the Environment to investigate the extent of environmental lead pollution and its effect on the health of the local population. Preliminary data, published in 1974, indicated that there had been a small increase (about 4–5 μg/100 ml) in the mean blood-lead concentrations of a group of adult men and women in the 2-year period since the opening of the motorway. Now a full report of subsequent studies has been published.

Measurements of atmospheric lead concentrations from a variety of sites revealed that the mean annual concentration of lead in air was less than the proposed EEC air quality standard of 2 μg/m^3. However, there was a distinct seasonal pattern and this level was reached on a monthly average basis in October, November and December. As expected the mean urban values were higher than those for the suburban area, but even so they were not abnormal in comparison with other large cities. An environmental assessment cleared other factors, including the use of lead-containing utensils, culinary aids or cosmetics, as potential sources of enhanced lead exposure. While the concentration of lead in dust samples from gutters was directly correlated with traffic flow, as would be expected, it was found that neither roadside noise-abatement barriers nor double glazing of house windows affected the general level of dust contamination.

In order to compare blood levels of lead, the city was subdivided geographically into four areas: an inner zone within the middle ring road, a mid-zone

between the middle and outer ring roads, an outer zone peripheral to the outer ring and finally an outside area, the whole of Sutton Coldfield. Within each of these areas, three population groups were sampled—adults, school children aged 8–14 years and pre-school children aged 1–5 years.

Equal groups of men and women (225 and 221 respectively) not occupationally exposed to lead were studied. Results indicated that the blood-lead concentration range and distribution for women (3–35 μg/100 ml) was within the reference concentration in the EEC Directive on Biological Screening of the Population for Lead, but for men there was an excess, the range being 9–46 μg/100 ml with a distribution shifted towards the higher values.

The mean blood-lead concentration in men, 22·1 μg/100 ml, was found to be unrelated to age, whereas the lower mean level in women, 14·7 μg/100 ml, increased with increasing age to reach the male population level in women about 40 years old. The blood levels were found to be higher in people living in older houses—a factor unrelated to the presence of lead pipes—and to be slightly increased in cigarette smokers. However the report strikes a note of caution on the interpretation of the data concerning the adult population which, it feels, was not representative of the adult city population in general, either geographically or ethnically.

The range and distribution of blood-lead concentrations in school children was similar to that in the adult female population, and none of the children examined had levels exceeding 35 μg/100 ml. The parental response to the study was not as great as had been hoped, and of 1120 children, consent was obtained for measurements on only 730. However, results showed that whereas there was no significant variation of mean blood-lead concentration with age in boys, in the girls the values were similar to those of the boys until the age of about 12 years, after which they were significantly lower. No difference between the mean blood concentration in the different racial groups was seen, but there was a significant decrease in values from the inner zone to the outermost areas, and a significant increase correlated with the age of the housing. Although the latter trend was irrespective of the presence of lead pipes, no environmental studies were carried out so the possible alternative sources of lead exposure are unknown.

A disappointing response was also obtained with pre-school children, under 5 years old, and only 243 of a total of 353 agreed participants eventually took part. Of these samples, the mean values were higher and the range was wider than was the case in blood from school children. Again the lowest mean values were in the outermost areas of the city. A disturbing feature of this part of the survey was the finding that 15 children had blood-lead concentrations equal to or greater than 35 μg/100 ml, and seven of these came from the total of 83 children examined in the two inner areas of the city. All but one of these children were of Asian origin. There was no consistent trend with housing age or with the habit of pica (ingestion of non-food materials) and follow-up studies to establish the source of lead exposure for young children living in the centre of the city have been initiated. Subsequent medical studies on nine of the children showing evidence of enhanced exposure revealed no frank neurological findings of significance; minor abnormalities in three of them could have been related to other factors.

In his foreword to the report the Minister of State at the Department of the Environment, the Rt Hon. Denis Howell, MP, summarizes the findings of the Working Party as showing nothing unusual as regards blood lead levels, nor any need for special concern about lead pollution under such traffic conditions. However, the report quite clearly states that the mean blood-lead concentrations of some residents has increased and that the latest work suggests that a relatively high proportion of very young children living in the inner city areas have elevated blood-lead concentrations. The atmospheric lead concentrations within the area do not appear to have risen concomitantly with the increase in traffic flow and due to practical difficulties encountered in carrying out the survey the Working Party concludes that it is not prepared to say whether the increase in blood-lead concentrations is a direct consequence of the increase in the traffic flow. However at the same time it does point out that while it cannot be certain that lead emitted in traffic exhaust caused the increase in blood levels, no alternative hypothesis can be put forward at present. The Working Party also concluded that the airborne lead levels around the motorway interchange are not exceptional by urban standards and that there is nothing unusual in the air and dust levels of lead by comparison with other large cities. Nevertheless the fact remains that lead is accumulated in the human body and any increase in the level of exposure cannot be ignored, especially in the very young, in whom active growth periods favour lead absorption. Even if the motorway system is exonerated by this report it is to be hoped that the new investigations recommended by the Working Party will be initiated.

La Réglementation des Produits. Alimentaires et Autres. Qualité et Répression des Fraudes. By R. A. Dehove. 9th Ed. Commerce Editions, Paris, 1978. pp. xvi + 1060. F.fr. 260.00.

The ninth edition of "Dehove" has now been published. This comprehensive documentation of French legislation concerning the sale of all merchandise, including food and drink, first appeared in 1954, and has been up-dated at regular intervals. The new edition follows the same format as that of its predecessor, reviewed some time ago (*Cited in F.C.T.* 1975, **13**, 569). It is thus similarly divided into six parts, starting with the Constitutional Law of 1 August 1905, which forms the basis of subsequent regulations, and ranging from food hygiene, packaging and labelling and the sale of food and beverages to animal foodstuffs and agricultural and household products.

Since the last edition appeared in 1974, the text has been considerably updated to incorporate not only the latest French legislation but also the most recent manufacturing and commercial information. However, perhaps the most important and useful addition, for non-French readers, is the inclusion where possible of the latest legislation resulting from Common Market directives. In particular the

chapters on sugar products and chocolates, wine and cosmetics have been extensively modified. Some minor changes have resulted from suggestions and requests from readers of previous editions and last, but certainly not least, the key index at the back of the book has been extensively revised to incorporate all the changes embodied within the text.

Always a welcome addition to any library, "Dehove" should prove in this new edition to be of wider scope and therefore of even greater value than before.

Health and the Environment. Public Health in Europe No. 8. WHO Regional Office for Europe, Copenhagen, 1977. pp. iii + 162. Sw.fr. 18.00 (available in the UK through HMSO).

Recent developments have dramatically extended the hostile factors man may encounter in his environment, adding many industrial chemicals, new forms of radiation exposure and other man-made hazards to established dangers such as those presented by disease organisms. The extent and speed of these developments constitute a major challenge to man's capacity for biological adaptation.

This volume in the 'Public Health in Europe' series examines some ways in which society is attempting to come to terms with both old and new health problems. It brings together contributions for 14 WHO Regional Office staff members and consultants and provides a useful indication of the organization's environmental activities within Europe. Clearly WHO is extremely active in fostering work designed both to maintain and to improve the European environment. The solutions are not restricted to theoretical generalization, as has often been the case in the past; examples are drawn from operational schemes dealing with problems such as noise pollution in Athens, air pollution in Bilbao and water pollution in Hungary.

Many of the problems are at their most acute in rural areas and developing countries. Such regions, in contrast to urban zones, have inherited a poor economy, are likely to be undergoing a population explosion and lack adequate materials and trained man-power. Their problems tend, therefore, to be self-perpetuating, and migration from rural to urban areas has often been a result. To some extent such problems may be eased by a co-ordinated international approach. Organizations other than WHO have been involved and mention is made of collaboration with the International Labour Office (ILO), the United Nations Development Programme (UNDP) and the International Agency for Research on Cancer (IARC).

For the industrial toxicologist, two of the contributions in this report should prove particularly interesting. Both are concerned with the problems of introducing new chemicals into industry and adopting exposure limits. Their approach is general, although they give a reasonable idea of the present state of affairs in Europe. We are reminded that judgements and decisions by health departments and other government organizations are seldom based solely on the available scientific evidence. Political, cultural and economic considerations cause local, regional and national authorities to come to quite different policy conclusions. Unfortunately, attempts to reconcile the different approaches to the setting of criteria and limits have progressed very slowly.

Despite the vast scale of the planning involved, one gets the impression that some progress is being made. Let us hope, therefore, that the lessons learned will be of value in the development of similar projects in the Third World.

BOOKS RECEIVED FOR REVIEW

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man: Some Aromatic Amines and Related Nitro Compounds—Hair Dyes, Colouring Agents and Miscellaneous Industrial Chemicals. Vol. 16. International Agency for Research on Cancer, Lyon, 1978, pp. 400. Sw.fr. 50.00.

Micro-organisms in Foods 1: Their Significance and Methods of Enumeration. 2nd Ed. International Commission on Microbiological Specifications for Foods. University of Toronto Press, Toronto, 1978. pp. xvii + 434. $30.00.

Carcinogenesis and Mutagenesis: Predictive Tools in Application to Safety Evaluation/The Mutagenic Properties of Chemicals/Report of the Subcommittee on Inhalation Toxicology. Edited by M. A. Mehlman, M. F. Cranmer and R. E. Shapiro. Pathotox Publishers, Inc., Park Forest South, IL, 1977. pp. x + 388. $18.50.

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Fd Cosmet. Toxicol. Vol. 17, pp. 171 to 174
Pergamon Press Ltd 1979. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

LEAD—A WEIGHTY PROBLEM

Lead is ubiquitous in nature, but the main source of the environmental lead problem stems from exploitation of these natural sources by man. Used since at least 3000 BC, the environmental concentration of lead has risen sharply since the advent of the industrial revolution. The hazard to man is now well documented and is continually under review. A recent survey of household water supplies in Great Britain, for example, revealed that levels higher than those recommended by WHO (*Cited in F.C.T.* 1972, **10**, 228) and specified by the more recent EEC draft directive (*Off. J. Europ. Commun.* 1975, **18** (C214), 2) were more widespread than had been expected (*Cited in F.C.T.* 1978, **16**, 278).

The atmospheric concentrations of lead in urban areas and particularly those found near congested road junctions have for long been a source of public anxiety, and the latest government enquiry on the possible environmental hazard of living near busy motorways has focussed on Birmingham's Spaghetti Junction (*Cited in F.C.T.* 1979, **17**, 167). An indication of an increase in the mean blood-lead level of some residents even in the absence of any alteration in airborne lead concentration will give rise to further thought.

Investigation of the health of various sectors of the public must necessarily be limited by their very nature, and studies that add to the knowledge of the absorption, excretion and metabolism of lead in animals will always be regarded with interest as a possible insight into the fate of lead in man. In a recent study, quantitative estimates of the absorption, excretion and metabolism of lead in rats were made by measuring total-body radioactivity in a small-animal whole-body liquid scintillation detector following administration of a radioisotope of lead (^{203}Pb or ^{210}Pb) after overnight starvation (Conrad & Barton, *Gastroenterology* 1978, **74**, 731).

In the absorption studies, lead was given as 1 μCi ^{203}Pb, with varying amounts of $PbCl_2$ as carrier, either by direct stomach catheterization or by injection into an isolated intestinal segment following laparotomy. The urethra was sutured to prevent urinary losses of absorbed lead, and in most experiments the bile duct was isolated. Total-body radioactivity was measured shortly after the lead administration and 4 hours later, when the rats had been killed and the isolated segments of gut had been removed for separate assay of retained radioactivity. The percentage of radiolead retained in the intestinal segments was then determined by comparison with initial whole-body radioactivity. For the excretion studies, 2 μCi ^{210}Pb in saline was injected into the dorsal vein of the penis and whole-body radiation was determined immediately after dosing and at set intervals (initially daily) thereafter. Radioactivity of excreta and blood specimens was also measured in some experiments.

It was found that absorption of lead occurred almost solely from the small intestine, where it entered the epithelial mucosal cells. Maximum absorption took place in the duodenum where the mucosal uptake and subsequent transport into the body was enhanced by bile. Uptake was maximal within 0·5 hour of administration of the test dose, whereas absorption into the body increased slowly over a period of 2 hours, after which it levelled out. These results suggest that saturation of an intestinal-mucosa acceptor limits the amount of absorption from the gut. However, this block appears to be only a relative one as the larger the dose the greater is the amount of lead entering the body; also the total body burden does not affect absorption.

Various other factors were found to affect the absorption of lead, however. Certain physiological states, including iron deficiency and the period of rapid growth that occurs in the young animal, enhance absorption, as do ascorbic acid and other nutritional constituents that increase the solubility of lead. Iron, zinc and cadmium decrease lead absorption, not by affecting solubility but by competition for the receptor sites in the intestinal mucosa.

Lead excretion from the rat was found to be biphasic in character, more than half of the dose being lost within a week of administration, the greater part in the first day. Subsequent loss occurred over a period of months. Of the lead excreted, two thirds was passed out in the urine and most of the remainder in the faeces. Bile appeared to play an important role in the loss from the gut.

Intravenous injection of lead resulted in very rapid clearance from the plasma, only 10% of a dose of 2 μCi ^{210}Pb being recoverable after 5 minutes. A relatively large amount of the lead in the blood was concentrated in the erythrocytes, a fact which, together with the rate of loss from these cells, suggested that they served as an important transport mechanism for lead deposition in other organs or for excretion. Deposition was found to occur mainly in the kidneys and liver, which act as the principal routes of excretion, but it is important to note that levels in bone slowly increased before remaining relatively stable with a slow rate of loss.

These results suggest that while lead is excreted from the body, this process is limiting and long-term accumulation of lead occurs primarily in the bone, where it is relatively unavailable for excretion.

A similar deposition of lead in kidneys and liver was found in experiments in neonatal rats (Stevenson *et al. Toxic. appl. Pharmac.* 1977, **40**, 161). Rat pups were given 50 μg lead daily by gastric intubation from

birth for 3 weeks, and this was followed by ingestion of 80 ppm lead in the drinking-water for a further 5 weeks. Throughout the 8-week experimental period, the concentration of lead in the renal tissue remained roughly constant; at week 8 it was twice as great as that in the liver where the level had dropped to approximately half the week-2 value. However, although no demonstrable lead deposition occurred in pulmonary tissue, thymidine incorporation into DNA showed an approximately two- to fourfold increase in the lung as well as in the liver and kidney tissues at the end of the 2 months. In all cases this was associated with a stimulation of the adenylate cyclase-cyclic AMP system of the tissues. Protein synthesis and cell growth were therefore found to be altered by lead but the actual presence of lead in the tissues concerned was apparently not essential. The fact that only trace amounts of lead were detectable in the lungs may well have been due to the clearance mechanisms in this organ.

The lung's two principal defence mechanisms against inhaled particles are the activity of the alveolar macrophages and the mucociliary escalator—the continual ciliary sweep of mucus-borne particles along the bronchial tract. Recent investigations on the effects of lead particles on these activities in the rat lung have been reported by Kaminski *et al.* (*Br. J. exp. Path.* 1977, **58**, 9). Lead oxide (PbO) was given in a single intrapulmonary dose of 0·25 or 1·0 mg suspended in 0·5 ml normal saline, quantities corresponding approximately to 1 and 4 mg/kg total body weight, respectively. The diameter of more than 79% of the PbO particles given this way was less than 5 μm. In rats killed 15 or 40 days after the intrapulmonary instillation, the particulate PbO was found to be deposited in the alveoli and alveolar macrophages. Large aggregates were seen occasionally but there was little evidence of lead in the interstitial tissues or regional lymph nodes.

Evidence of lead excretion from the respiratory tract via the mucociliary mechanism was obtained from examination of sections of bronchial tissue. These showed plaques of mucous material with lead-containing macrophages. This process obviously continued for some time.

When the macrophages obtained from the rats exposed to PbO were compared with those from the control groups, an increase in the number of viable cells recovered was found to follow the lead treatment. This was especially marked after treatment at the higher dose level, when the increase in viable cells was about 20-fold. This high level was maintained throughout the 40-day experimental period, but the *in vitro* viability of the cells in a culture medium was lower than that of the macrophages obtained from untreated rats, although this reduction was not dose dependent. These results indicate that the toxicity of PbO to alveolar macrophages is relatively low—intracellular PbO appears to be less toxic to macrophages than other fibrous particulates such as asbestos.

Lead is a vital metal for an industrial economy. Over the last 20 years, its consumption has increased in the western world at the rate of approximately 3·25% per year, and this trend is expected to continue (Robinson, in *The Biogeochemistry of Lead in the Environment*, Part A, edited by J. O. Nriagu; p. 99; Elsevier/North-Holland Biomedical Press, Amsterdam, 1978). While lead can be stored to a certain extent in the body tissues, the amount accumulated will obviously increase with age, and any addition to the total body burden by general environmental exposure must lead to concern.

[R. Hawkins—BIBRA]

FOETAL HEALTH WARNING

We last reviewed in April 1978 the literature on the effects that smoking during pregnancy may have upon the foetus (Cooper, *Fd Cosmet. Toxicol.* 1978, **16**, 187). During the intervening year, evidence of adverse effects has continued to accumulate.

A recent New York study lends strong support to the theory that cigarette smoking during pregnancy may increase the chances of spontaneous abortion (Kline *et al. New Engl. J. Med.* 1977, **297**, 793). The smoking habits of 574 women who had aborted spontaneously were compared with a control group of 320 women who gave birth after at least 28 weeks gestation. There were no significant differences between these two groups in age, marital status, ethnic group, place of birth or education. Women who had aborted spontaneously reported smoking during their pregnancy more often than those with delivery after 28 weeks, the figures being 41% for the former and 28% for the latter. When confounding variables were controlled in the analysis, women who suffered spontaneous abortions were twice as likely to have smoked. Commenting on these findings, a Leading Article in the *British Medical Journal* (1978, **1**, 259) emphasized that while earlier reports had reached similar conclusions, the New York survey was important in that it was conducted at a time when induced abortion was legally available. It was thus more likely that the observed abortions were truly spontaneous and not misreported illegally-induced ones.

In 1960–61 all mothers admitted to the maternity departments of ten Ontario teaching hospitals were interviewed soon after they had given birth and asked about their smoking habits during pregnancy. The resulting data, involving some 51,490 births including 701 foetal and 655 neonatal deaths, were analysed by Meyer & Tonascia (*Am. J. Obstet. Gynec.* 1977, **128**, 494). The analysis, which took account of the effects of maternal height, pre-pregnant weight, "hospital pay status" and age, previous pregnancies and the sex of child, showed that perinatal mortality increased by approximately 20% amongst smokers of less than one pack of cigarettes a day and 35% amongst those who smoked more than one pack a day. Controlling these same factors, it was found that

maternal smoking also had a significant, independent influence on the risks of premature delivery, placenta praevia and abruptio placentae and on the proportion of babies weighing less than 2·5 kg. Meyer & Tonascia (*loc. cit.*) calculated that between one third and one half of the increase in perinatal deaths found in the smoking mothers could be accounted for by the smoking-related increase in these complications.

An association between maternal smoking and early foetal death, particularly death caused by abruptio placentae, was also demonstrated by Goujard *et al.* (*ibid* 1978, **130**, 738) in their survey of 9169 births at a number of Paris hospitals during the period 1963–69. Both drinking and smoking during pregnancy were independently associated with an increased rate of stillbirth, the risk being roughly 2·5 times greater in each case. When considering only abruptio placentae as a cause of death, a six-fold risk was associated with tobacco smoking and there was a 30-fold risk for women who both smoked during pregnancy and consumed more than 45 ml of alcohol daily.

The results of a prospective study of 53,518 pregnancies seen in 12 US hospitals between 1959 and 1966 suggests that cigarette smoke is teratogenic (Naeye, *Am. J. Path.* 1978, **90**, 289). During this period, congenital malformations were responsible for 9·3% of all the perinatal deaths. At all gestation ages, there was a significantly higher rate of death due to this cause in mothers who had smoked between 11 and 20 cigarettes/day during their pregnancy than in those who had smoked less or not at all. Even with this huge sample size, numbers were inadequate for an analysis of the relationship of potential teratogenic factors to specific malformations, with one exception: an incidence of anencephaly of 1·72/1000 births in white subjects who smoked was significantly higher than the frequency of 0·10/1000 births seen in white non-smokers. Although one retrospective study had suggested an association between cigarette smoking during pregnancy and congenital heart disease in the offspring, independent of maternal age, parity and social class (*Cited in F.C.T.* 1973, **11**, 673), a number of other studies have failed to show any link between smoking and congenital abnormalities. It must be remembered, however, that the very large number of births that must be reviewed before any sound conclusions can be drawn on changes in malformation incidence makes this a particularly difficult area of study.

In spite of the number of reports of an association between cigarette smoking and reduced birth weight, there is still debate as to whether cigarettes are a direct cause of this condition. The view that the observed association was only indirect and that reduced birth weight was more a reflection of parental lifestyle than smoking itself (*Cited in F.C.T.* 1973, **11**, 671) has received renewed support from a small study by Alvear & Brooke (*Lancet* 1977, **I**, 1158), who found that infants born to women of social class IV or V who had smoked during pregnancy were comparable in size to infants born to an equal number of non-smoking women (106) of the same socio-economic class. The publication of this result generated the inevitable response from those convinced that smoking is a direct cause of reduced foetal weight. Murphy *et al.* (*ibid* 1977, **II**, 36) re-stated the conclusions they had drawn from their earlier published work, namely that smoking during pregnancy did have a significant effect on the size of the resulting foetus independent of the effect of social class. In their most recent study, as yet unpublished in full, a similar result was evidently found. The 21 babies born to smokers weighed 3·26 ± 0·36 kg, while 27 babies born to non-smokers weighed 3·69 ± 0·42 kg, the difference being statistically significant. Both groups were controlled for age, parity, length of gestation and socio-economic status.

An investigation of the obstetric history of over 7600 women doctors (Alberman *et al. ibid* 1977, **II**, 36) also contradicted the social class/lifestyle theory. In 1975 a questionnaire was sent to all women who were on the Medical Register and had qualified since 1950; almost three quarters of those contacted responded. At 3·41 kg, the average weight of the 6004 infants born to the mothers who had not smoked was significantly higher than the 3·33 kg recorded for the 1528 infants of mothers who had smoked during their pregnancy. Both groups would have been very similar in income and education.

The accumulating epidemiological evidence against smoking in pregnancy is also being supplemented by clinical data. Spira *et al.* (*Biomedicine* 1977, **27**, 266) studied the pathology of the placentas of 248 women who had smoked at least five cigarettes a day during pregnancy, and of 196 non-smoking controls. There were no significant differences in the placentas of the two groups on macroscopic observation, but when examined microscopically, the placentas of smokers showed a significantly higher frequency of trophoblast abnormality and especially of nuclear clumps in the syncytiotrophoblast. Only 6% of the placentas taken from non-smokers showed any features that could be considered indicative of hypoxia, a proportion significantly lower than the 16% incidence amongst the placentas of smokers. Placental weights of both groups were comparable, in spite of the lower weights of the foetuses from smoking mothers. Furthermore, the foetal weight showed no direct relation to the presence or absence of signs of hypoxia. The authors considered that the intra-uterine hypoxia within the smoking group could explain the increased risk of placenta praevia, abruptio placentae and perinatal death previously observed amongst smokers. On the other hand they sugggested that the reduced birth weight might reflect a functional disorder within the placenta.

To add to this sad tale, some experiments showing that placental microsomes of smokers had a greater ability to metabolize benzo[*a*]pyrene than had those of non-smokers led Wang *et al.* (*Life Sci.* 1977, **20**, 1265) to suggest that maternal smoking during pregnancy might increase the susceptibility of the offspring to cancer after birth. Placental microsomes from ten of 11 non-smokers did not metabolize benzo[*a*]pyrene *in vitro* significantly. The microsomal fraction of the placenta of six smokers, by contrast, metabolized this polycyclic aromatic hydrocarbon to a number of metabolites, some of which may be carcinogenic. However, the total quantity of metabolites showed no clear relationship to the number of cigarettes smoked, and there appears to be no direct evi-

dence that smoking in pregnancy increases the incidence of cancer in the offspring.

In an earlier review (*Cited in F.C.T.* 1975, **13**, 381) we mentioned a study in which significant differences in height and reading ability were found between the 7-year-old children of smokers and non-smokers. A small prospective study conducted in the USA, involving 325 children in all, has now produced similar findings (Dunn & McBurney, *Pediatrics, Springfield* 1977, **60,** 772). When 6·5 years old, the children of non-smokers had a slightly greater average height and weight and performed better in psychological tests than did those of smokers. Variations in social class between the two groups could only partially account for the observed differences.

In epidemiology, other things being equal, the larger the sample size the more significant is the study. A number of environmental factors may have a detrimental effect on the course of a pregnancy, so large samples are essential for unravelling the effect of a single parameter. The problems inherent in considering only a small sample can be demonstrated by the data of Alberman *et al.* (*loc. cit.*) If the small sub-group of mothers who had had three pregnancies are considered alone, the average weight of the non-smokers' offspring was 3·47 kg (439 children) whereas the average weight of the 109 infants born to the smokers was 3·55 kg (109 children): the overall findings of the study were therefore not demonstrated by this small sample.

Current research concerned with the health of the passive smoker, of which the foetus is surely a prime example, is suggesting the need for education on the broader effects of smoking. The studies reviewed above lend further support to the contention that a mother who smokes may be seriously jeopardizing her unborn child, and a forceful campaign to inform those directly involved of the possible adverse effects of smoking in this context does seem to be warranted.

[J. Hopkins—BIBRA]

Fd Cosmet. Toxicol. Vol. 17, pp. 175 to 181
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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

COLOURING MATTERS

Dietary fibre—food for dyes?

Ershoff, B. H. (1977). Effects of diet on growth and survival of rats fed toxic levels of tartrazine (FD & C Yellow No. 5) and Sunset Yellow FCF (FD & C Yellow No. 6). *J. Nutr.* **107,** 822.

The capacity of plant fibre to protect experimental animals from the toxicity shown by certain chemicals and food additives when fed with a purified diet is now fairly well documented. Previous findings include a protective role against the toxicity of amaranth (FD & C Red No. 2) fed at a 5% level in a purified diet (*Cited in F.C.T.* 1975, **13,** 581) and of sodium cyclamate and polyoxyethylene sorbitan monostearate at 5 and 15% levels respectively in low-fibre diets (*ibid* 1976, **14,** 365).

The protective effect of dietary fibre has now been demonstrated following administration of the water-soluble azo dyes, tartrazine (FD & C Yellow No. 5) and Sunset Yellow FCF (FD & C Yellow No. 6). Immature male rats fed either dye at a 5% level in a stock diet based on common feed ingredients showed no grossly observable toxic effects, but within a 14-day experimental period marked growth retardation, poor fur and death of 50% or more animals resulted when these levels were incorporated in a purified diet (66% sucrose, 24% casein, 5% salt mixture, 5% cotton-seed oil and vitamins).

Little if any protective effect resulted from supplementation of the purified diet with additional vitamins, 2·5% salt mixture, 5% cotton-seed oil and 10% casein, either singly or combined, or with 10% desiccated whole liver. Cellulose at the 2·5, 5 and 10% levels was without effect when incorporated into the purified diet containing 5% tartrazine, although it did have a small but significant effect in the purified diet with 5% Sunset Yellow FCF.

However, the toxic effects of Sunset Yellow FCF at the 5% level in purified diet were completely counteracted by concurrent administration of a 10% level of blond psyllium-seed powder or carrot-root powder and were counteracted to a slightly less marked degree by a 10% level of alfalfa leaf meal or wheat bran. All four of these fibre-containing supplements largely counteracted the toxic effects of tartrazine at a 5% level in the purified diet.

The factor(s) involved and the possible mechanism of action are as yet unknown. It has been suggested that the protective effect of dietary fibres is probably due to their physico-chemical properties rather than to their chemical content, and further, in view of the poor protection afforded by cellulose, that hemicellulose and/or other plant ingredients play the major role. The binding of the food colourings or their metabolites, with consequent inhibition of absorption, is one possible mechanism, particularly in view of the anion-exchange activity of the supplements tested, but it is pointed out that the prevention of adverse effects of the colouring on the digestive processes or the gut bacteria cannot be excluded.

FLAVOURINGS, SOLVENTS AND SWEETENERS

Eugenol plays on the nerves

Kozam, G. (1977). The effect of eugenol on nerve transmission. *Oral Surg.* **44,** 799.

Eugenol is the main constituent of essential oils such as oil of clove, clove stem and leaf, pimenta berry and leaf, bay and cinnamon leaf. It also occurs in smaller quantities in many other oils. The compound has been used as a fragrance and is also used widely in dental practice as a disinfectant in root-canal procedures and as a local anodyne in the relief of hyperaemic and inflamed pulp. A number of problems have arisen from the dental use of eugenol. Waerhaug & Loe (*Oral Surg.* 1957, **10,** 923) observed a necrotic reaction under eugenol gingival packs, whilst Guglani & Allen (*J. Periodont.* 1965, **36,** 279) observed subcutaneous inflammation resulting from periodontal pack implants. In addition, the compound has been shown to possess allergenic properties (Koch *et al. Odont. Revy.* 1971, **22,** 275; *idem, ibid* 1973, **24,** 109). The above-cited study examines the effects of eugenol on nerve transmission, and it is suggested that the type of test used may be the most appropriate for testing endodontic medications.

Frog sciatic nerve was exposed to varying concentrations (0·005–100%) of eugenol and the oscilloscopic findings of the effects were measured, photographed and recorded over a 3-hr period. Four experiments were conducted for each concentration of eugenol tested. Six control runs were made with oil of sweet almond before the experimental series was initiated and four more were carried out during the experimental programme.

Eugenol concentrations between 100 and 0·1% had a definite blocking effect on the transmission of evoked impulses in nerve tissue. Concentrations of 100, 50 and 25% had about the same effectiveness, whilst lower concentrations had less effect and showed a direct dose–response relationship. The time required for the action potential to be extinguished increased progressively as the concentration was reduced from 25 to 0·05%. Tests conducted with 0·005% produced neither reduction in amplitude nor changes in configuration of the compound action potential. No recovery of any amplitude lowered or

extinguished by eugenol was ever noted in the 3-hr observation period, although the possibility that recovery might have occurred at a later time could not be excluded.

The author considers that there is now adequate evidence to indicate that eugenol can have toxic effects when used improperly for endodontic therapy. This would be especially so at concentrations that are currently available commercially (90–100%) and caution in use is therefore recommended.

Aspartame and the blood

Stegink, L. D., Filer, L. J., Jr. & Baker, G. L. (1977). Effect of aspartame and aspartate loading upon plasma and erythrocyte free amino acid levels in normal adult volunteers. *J. Nutr.* **107,** 1837.

Aspartame (L-aspartyl-L-phenylalanyl methyl ester), which has a sweetening potential some 200 times that of sucrose, has been suggested as a low-calorie sweetener for foods (*Food Chemical News* 1973, **14** (49), 14). It has been reported not to affect the metabolism of phenylalanine in monkeys (*Cited in F.C.T.* 1974, **12,** 778) and to be well tolerated by mildly affected phenylketonuric adolescents (*ibid* 1978, **16,** 77).

In studies of the effect of single loading doses of aspartame and aspartate on the amino acid composition of blood, 12 healthy adults were given, in orange juice, 34 mg aspartame/kg and an equimolar dose of 13 mg aspartate/kg in a crossover trial, and plasma and erythrocyte amino acids were determined at intervals from 0·25 to 24 hr after ingestion of the dose. Neither aspartame nor aspartate produced any change in the aspartate levels in plasma and erythrocytes. Aspartame increased plasma- and erythrocyte-phenylalanine concentrations to peak values of 11·1 μmol/100 ml and 7·21 μmol/100 g respectively after 30–60 min. The values returned to baseline within 4–8 hr. Aspartate reduced plasma concentrations of phenylalanine slightly. Plasma-tyrosine levels, indicating the degree of conversion of phenylalanine, increased slightly after aspartame ingestion and decreased slightly after aspartate. The findings suggest that, as previously indicated (*ibid* 1978, **16,** 293), aspartame is readily metabolized in man, with rapid elimination of its metabolites. The data reported are consistent with those derived from more prolonged administration of the sweetener to healthy subjects (Frey, *J. Toxicol. envir. Hlth* 1976, **2,** 401) and others (*Cited in F.C.T.* 1978, **16,** 77; Stern *et al. J. Toxicol. envir. Hlth* 1976, **2,** 429).

Saccharin excretion

Goldstein, R. S., Hook, J. B. & Bond, J. T. (1978). Renal tubular transport of saccharin. *J. Pharmac. exp. Ther.* **204,** 690.

Considerable attention has been given to the concern for the safety of saccharin and to the recommendations for further research to elucidate its potential role as a bladder carcinogen (*Food Chemical News* 1977, **19** (9), 2; *ibid* 1978, **19** (46), 65). Saccharin is rapidly excreted from the body, and the actual mechanism of renal transport has now been examined in the female rat, both *in vitro* using renal cortical slices and *in vivo* using renal clearance.

Incubation of renal cortical slices with saccharin demonstrated that saccharin accumulation depends upon the concentration. At concentrations greater than 10^{-4} M there was a progressive decrease in the accumulation, suggesting that saccharin was being accumulated against a concentration gradient in a saturable system. These observations, together with the dependence of the system upon the presence of oxygen, led to the conclusion that uptake of saccharin is an active transport system. Other characteristics demonstrated were a reduction in accumulation in the presence of metabolic inhibitors (2,4-dinitrophenol, an uncoupling agent for oxidative phosphorylation, and sodium azide, a respiratory-enzyme inhibitor) and of *p*-aminohippurate (PAH) and probenecid. Uptake was stimulated by addition of acetate and lactate to the incubation medium, but decreased by reducing the amount of available potassium.

The authors postulated from these observations that saccharin transport might well share the same biochemical requirements as PAH, and found that indeed saccharin produced a dose-related depression of PAH accumulation and *vice versa*, indicating a competitive uptake by a common transport system. However, an unexplained finding was the non-dose-related depressive effect of saccharin on accumulation of the organic base *N*-methylnicotinamide.

Renal-clearance experiments following venous infusion of inulin (to provide a measure of the glomerular filtration rate) indicated that the primary route of renal elimination of saccharin is active tubular secretion. This again is similar to the findings with PAH, enhancing the authors' belief that both compounds share a common transport system.

AGRICULTURAL CHEMICALS

A clean sheet for dieldrin handlers

Van Raalte, H. G. S. (1977). Human experience with dieldrin in perspective. *Ecotoxicol. envir. Safety* **1,** 203.

Opinions vary widely on the extent to which organochlorine pesticides may represent a threat to public health, and any potential risk has to be weighed against the considerable benefits offered by this group of compounds. While arguments continue on the possible significance of some of the effects demonstrated in animals, vigilance is necessary to ensure that any adverse effect induced in man is promptly detected. In this connexion, the regular observation of workers handling such pesticides is of major importance.

The follow-up results of a long-term Dutch study of industrial workers handling aldrin and dieldrin have been reviewed against a background of animal studies, including those demonstrating the induction

of hepatic carcinomas in mice but not in other experimental animals and the similarity between liver responses to dieldrin and to phenobarbitone. During the period 1954–67, there were 32 instances of clinical intoxication involving dieldrin at the insecticide manufacturing and formulation plants at Pernis; 19 of these patients had convulsions. The average blood concentration of dieldrin when convulsions occurred was 280–290 ppb ($b = 10^9$) and no case of convulsions was associated with blood levels of dieldrin below 200 ppb. In some apparently healthy workers, however, blood-dieldrin concentrations exceeding 400 ppb were detected. The no-effect blood level of dieldrin in 233 men was established at 200 ppb, which corresponded to a long-term intake of 33 μg/kg/day. No signs of microsomal-enzyme induction by dieldrin were demonstrated by measurements of the blood concentrations of DDE, the ratio of 6-β-hydroxycortisol to 17-hydroxycorticosteroids in the urine and the urinary excretion of D-glucaric acid. There was no evidence of an excess incidence of tumours among the Pernis workers, and no toxic changes suggesting the likelihood of a later development of tumours were detected.

Ethylene thiourea, a mixed mutagenic picture

Schüpbach, M. & Hummler, H. (1977). A comparative study on the mutagenicity of ethylenethiourea in bacterial and mammalian test systems. *Mutation Res.* **56,** 111.

Teramoto, S., Moriya, M., Kato, K., Tezuka, H., Nakamura, S., Shingu, A. & Shirasu, Y. (1977). Mutagenicity testing on ethylenethiourea. *Mutation Res.* **56,** 121.

Ethylene thiourea (ETU) is a decomposition product of ethylene-bis(dithiocarbamate) biocides and has been shown to be mutagenic in bacteria (*Cited in F.C.T.* 1975, **13,** 398) and teratogenic in rats (*ibid* 1973, **11,** 702 & 419).

In the first paper cited above, ETU (20 mg or more/plate) is reported to have produced a significant and dose-dependent increase in prototrophic revertants of *Salmonella typhimurium* strain TA1530 in agar culture, but was ineffective in strains reverted by frameshift mutagens. The ability of ETU to induce mutations of the base-pair substitution type was also demonstrated in a host-mediated assay in mice, but only with a dose of 6000 mg/kg in a strain lacking a fully active repair system. This dose caused a slight (2·37-fold) but significant increase in reversion frequency. In a micronucleus test (*Cited in F.C.T.* 1977, **15,** 646) in mice, two oral doses each of 6000 mg ETU/kg had no significant effect on bone-marrow erythrocytes, and single oral doses of 500–3500 mg ETU/kg given to male mice failed to produce any results that were considered to reflect a dominant-lethal effect.

These results, although indecisive, were to some extent supported by the work outlined in the second paper cited. No growth inhibition occurred in *Bacillus subtilis* strains of H17 Rec^+ or M45 Rec^- during exposure to ETU in concentrations of 20–4000 μg/plate. Reversion assays using *Escherichia coli* WP2 and five strains of *S. typhimurium* again indicated that ETU was a weak base-pair mutagen, inducing reverse mutations in TA1535 at concentrations of 5000 μg/plate or more; with 20 mg ETU/plate there was a four-fold increase, but TA100 was unaffected even at this level. A host-mediated assay in rats and mice showed no increase in mutation frequency in *S. typhimurium* G46, whereas nitrosodimethylamine used as a positive control showed high mutagenicity, particularly in mice. Cytogenetic studies of ETU in Chinese hamster Don cells and rat bone-marrow cells demonstrated no mutagenicity, and mice given 300 or 600 mg ETU/kg orally for five consecutive days showed no increase in dominant-lethal mutations.

The importance of ETU as an environmental contaminant makes it a fertile field for study, and the experiments reviewed here suggest the need for further clarification of the question of ETU mutagenicity. The nitrosated derivative of ETU is reported to be undergoing cytogenetic and dominant-lethal studies, interactive mutagenicity of ETU and nitrite having been demonstrated in a host-mediated assay (Shirasu *et al.* Cold Spring Harbor Conferences on Cell Proliferation, Vol. 4; Cold Spring Harbor Laboratory, 1977).

Nitrosamines in herbicides too?

Ross, R. D., Morrison, J., Rounbehler, D. P., Fan, S. & Fine, D. H. (1977). *N*-Nitroso compound impurities in herbicide formulations. *J. agric. Fd Chem.* **25,** 1416.

The likelihood that nitroso compounds are formed by the reaction of some types of agricultural compounds with nitrites has previously been explored (Elespuru & Lijinsky, *Fd Cosmet. Toxicol.* 1973, **11,** 807). Preformed nitroso compounds have now been detected in some herbicide formulations.

Nitrosodimethylamine (NDMA) was determined in six herbicide formulations and nitrosodipropylamine (NDPA) in one, using gas chromatography–thermal energy analysis or high-pressure liquid chromatography–thermal energy analysis. In the first six herbicide formulations all the listed constituents were present as dimethylamine salts. Two formulations containing 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,6-dichloro-*o*-anisic acid were tested; the first also contained 2-(2-methyl-4-chlorophenoxy)propionic acid. NDMA was detected, at 0·3 ppm, in this first sample but not in the second sample. No NDMA was found in a third sample made up of 2,4,5-trichlorophenoxypropionic acid and 2,4-D. Three formulations of 2,3,6-trichlorobenzoic acid had the highest NDMA levels, of 187, 195 and 640 ppm respectively. The seventh sample, a formulation of α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine, was analysed for NDPA and found to contain 154 ppm. The authors speculate that NDPA may result from the nitrosation of dipropylamine by nitric acid, since both of these compounds are used in the preparation of α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine. In the case of the 2,3,6-trichlorobenzoic acid samples, the NDMA may have resulted from the reaction of dimethylamine with sodium nitrite used in the containers as a rust inhibitor.

The potential routes of human exposure to herbicides are numerous. The authors of this paper point out that concentrations of NDMA as high as 640 ppm in herbicides could lead to human exposures much higher than those from nitrite-preserved foods or tobacco smoke. They suggest that epidemiological studies should be carried out on people exposed to high levels of herbicides.

PROCESSING AND PACKAGING CONTAMINANTS

Assessing plastics migration

Rossi, L. (1977). Interlaboratory study of methods for determining global migration of plastic materials in liquids simulating fatty foodstuffs. *J. Ass. off. analyt. Chem.* **60,** 1282.

The overall migration into foods of the constituents of plastics materials with which the foods are in contact is already limited by law in some countries (e.g. France and Italy). In addition a proposed Directive has been placed before the EEC Council (*Off. J. Europ. Commun.* 1978, **21** (C141), 4). This proposal would, in most cases, limit migration to 60 mg/kg of food or 60 mg/litre of liquid. The methods of analysis to be used will be set out in a further directive. The paper cited above was first presented in November 1976 and describes the work of an EEC Working Party responsible for assessing possible methods for determining overall migration of plastics constituents into fatty foods. The author compares the results obtained by the various methods in a number of laboratories.

The methods used to determine migration of plastics into fats are all based on the same principles. The plastics sample is weighed before and after contact with the fat simulant, and the amount of simulant absorbed by the sample is extracted and determined; the plastics migration can then be estimated from these three weights. The methods differ in the choice of simulant and in the technique used for determining the absorbed simulant.

Initial comparison of three possible methods led to the rejection of the Pallière (*Annali Ist. sup. Sanita* 1972, **8,** 365) method, in which the simulating liquid used is sunflower oil which is extracted and determined iodometrically. Although this method was found to be simpler and quicker than the other two methods, it was hampered by interference from unsaturated substances that could co-extract with absorbed oil from the plastics material. The methods of Van Battum & Rijk (*ibid* 1972, **8,** 421) and of Rossi *et al.* (*ibid* 1972, **8,** 432) were not subject to interference and were considered worth further investigation, although the reproducibility between laboratories was poor. These two methods used HB 307 (a synthetic mixture of triglycerides) and sunflower oil, respectively, as simulating liquids; in both cases the determination was made by gas chromatography. On the basis of these first interlaboratory tests, the Working Party prepared a new method—'the Community method' (EEC Working Document No. 3003/VI/74, chap. III, p.34). Unlike the procedures discussed above, this new method specified olive oil as the simulating liquid, since it was more stable than sunflower oil and less expensive and easier to use than HB 307. The Community method was used in five different laboratories and was judged to be valid for low-density polyethylene, rigid PVC, polystyrene and polyamide, but not for acrylonitrile–butadiene–styrene copolymer; reproducibility was considered satisfactory "if the fact that the laboratories had not adhered strictly to the technical details was taken into account". However, the general validity of the method was doubted by one of the laboratories, after the observation that the fatty acid composition of the olive oil was different before and after extraction. Subsequently the Community method was further tested and compared with a modified Van Battum method (EEC Working Document No. 1889/VI/75) and with the Figge method which used labelled HB 307 as simulant and a radiometric determination (*Bundesgesundheitsblatt* 1975, **18,** 27). No significant interferences in the quantitation of the oil were found for any of these methods. Variations in the composition of the olive oil did not appear to affect the final result. All three methods gave "acceptable" results for the plastics examined, namely polyethylene of various melt indexes, polypropylene, plasticized and rigid PVC, crystalline and high-impact polystyrene and acrylonitrile–butadiene–styrene copolymer. Reproducibility within a laboratory was fairly good for the Community and Figge methods but slightly less for the Van Battum method. Variations between laboratories were much greater than those within a laboratory, but here again the Community and Figge methods showed better reproducibility than the Van Battum method. The Figge method was more reliable than the other two methods in respect of the possible variations in oil composition and of the presence in the extract of substances interfering with gas chromatography. However, these advantages are said to have little practical value at present. It is also pointed out that the Figge method needs special precautions and specialized personnel and apparatus which may not be available in all laboratories.

THE CHEMICAL ENVIRONMENT

Cadmium and liver pathology

Faeder, E. J., Chaney, S. Q., King, L. C., Hinners, T. A., Bruce, R. & Fowler, B. A. (1977). Biochemical and ultrastructural changes in livers of cadmium-treated rats. *Toxic. appl. Pharmac.* **39,** 473.

The toxicity of cadmium and cadmium compounds is now well documented; exposure for any length of time results in accumulation and subsequent damage to both the liver and kidneys. These two organs together account for about half of the body's burden of cadmium following prolonged exposure (*Cited in*

F.C.T. 1977, **15**, 480). Although the evidence for any teratogenic or carcinogenic activity of cadmium is considered to be inconclusive, concern for industrial safety has led NIOSH to recommend a lower TLV of 40 $\mu g/m^3$ rather than the previous level of 50 $\mu g/m^3$ (*Federal Register* 1977 **42**, 5434).

Liver damage can be detected directly by electron microscopy, and also indirectly by improper function, as reflected by abnormal plasma-enzyme levels. Exposure to cadmium in the work now reported was regulated so that the doses administered would produce liver change, but injections were timed to prevent development of obvious morbidity. Male rats were injected sc with cadmium chloride in saline in doses of 0, 0·25, 0·50 or 0·75 mg cadmium/kg body weight three times/wk over a period of 8 wk. Every 2 wk representative animals from each dose level were killed and cadmium levels were determined. Change in liver function was measured by assay of plasma levels of aspartate aminotransferase (AAT), γ-glutamyl transpeptidase (GT) and ornithine carbamoyltransferase and by determination of red-cell carbonic-anhydrase activity. Liver structure was examined by electron microscopy.

It was found that cadmium accumulated in the liver, increasing in all dose groups as a function of both time of treatment and dose. Between 40 and 50% of the total body burden of cadmium was found to be accumulated in the liver, while kidney levels accounted for between 5 and 8% of the total. Liver function tests revealed that by wk 6 both AAT and GT levels had become elevated in rats given the higher doses. Also at wk 6 a small but significant decrease in the red-cell carbonic-anhydrase activity occurred in the two highest dose groups and changes in the cellular structure became apparent. Examination of liver tissue with the electron microscope revealed a dilation and loss of ribosomes from the endoplasmic reticulum and a proliferation of large connective tissue bundles which had the appearance of newly formed collagen fibres.

A definite correlation was therefore established between the onset of ultrastructural change in the liver and biochemically observable enzyme-level change.

The authors suggest, on the basis of these findings, that while the concentration of cadmium is important, the rate of its accumulation is another important factor governing morbidity. When the rate of cadmium exposure is slow, sufficient protein can be synthesized for liver damage to be prevented or minimized.

Mutagenicity of acrylonitrile

Milvy, P. & Wolff, M. (1977). Mutagenic studies with acrylonitrile. *Mutation Res.* **48**, 271.

Venitt, S., Bushell, C. T. & Osborne, M. (1977) Mutagenicity of acrylonitrile (cyanoethylene) in *Escherichia coli*. *Mutation Res.* **45**, 283.

The monomer acrylonitrile (CH_2 = CHCN; vinyl cyanide; propenenitrile; cyanoethylene; ACN) is produced industrially on a large scale and is widely used in the manufacture of rubber and synthetic fibres and also as a grain fumigant. A preliminary report has indicated that ACN may be carcinogenic in rats (*Food Chemical News* 1977, **19**(4) 23) and in a study of workers in a polymerization plant has suggested a possible carcinogenic hazard in industrial situations (*ibid* 1977, **19** (11) 26). Furthermore, two papers published within weeks of each other have indicated the mutagenic properties of ACN, in one experiment at a vapour-phase exposure level as low as 57 ppm.

Milvy & Wolff (cited above) demonstrated mutagenesis using the Ames *Salmonella typhimurium* strains TA1535, TA1978 and TA1538 exposed to ACN under three different experimental conditions (solution, vapour and spotting on a bacterial 'lawn'). Of the three techniques used, bacterial exposure to ACN in the vapour phase was found to be the most useful for quantitative studies owing to the compound's high volatility. Increases in both base-substitution and frameshift mutations were observed only in the presence of microsomal-enzyme systems and co-factors —in this work the S-9 fraction from rat-liver homogenate.

Mutagenicity of ACN was also demonstrated by Venitt *et al.* (*loc.cit.*), using *Escherichia coli* WP2 strains in plate-incorporation and fluctuation tests. The latter technique detected mutagenic activity in the WP2 *uvrApolA* strain at a concentration of 0·1 M × 10^{-3} ACN. The use of *S. typhimurium* strains *his*G46, *his*3052, TA1535, TA100, TA1538 and TA98 in the fluctuation test only demonstrated mutagenic activity in the *his*G46 strain, but these results were erratic and not significant. However, from the differential response of the *E. coli* WP2 series used, it appears that ACN may react with thymine residues in DNA.

[Although the majority of known chemical carcinogens have proved mutagenic in bacterial test systems, danger lies in extrapolating data from a mutagenic to a carcinogenic potential. Moreover the step from microbial observations to activity in man is a large one. Nevertheless these results do encourage further consideration of a possible ACN hazard to man in occupational exposure.]

Acrylonitrile in action again

Szabo, S., Bailey, K. A., Boor, P. J. & Jaeger, R. J. (1977). Acrylonitrile and tissue glutathione: differential effect of acute and chronic interactions. *Biochem. biophys. Res. Commun.* **79**, 32.

The preliminary indications of the possible carcinogenic potential of acrylonitrile in rats (*Food Chemical News* 1977, **18** (45), 20) and industrial workers (*ibid* 1977, **19** (11), 26) have led to the tightening of restrictions governing the use of this chemical. An emergency temporary standard for acrylonitrile has been issued by OSHA, reducing the permissible industrial exposure level from 20 to 2 ppm, as an 8-hr time-weighted average (*Federal Register* 1978, **43**, 2586).

Further data on the toxicity of acrylonitrile in rats is presented by the authors cited above. The same group had previously demonstrated that a single dose of acrylonitrile rapidly produced a fatal adrenal apoplexy in rats following adrenocortical insufficiency, central nervous system toxicity and congestive lung oedema (Szabo *et al. Am. J. Path.* 1976, **82**, 653). The

more recent paper reports the effects of acrylonitrile on reduced glutathione (GSH). The tripeptide glutathione occurs widely in animal tissues, and in its reduced form (GSH) it seems to act as an antioxidant in protecting the liver or lung from chemical injury.

In acute experiments, rats were given either an aqueous suspension of acrylonitrile (one iv dose of 150 mg/kg) and killed 5, 15, 30 and 60 min after treatment, or an iv injection of 0, 10, 50 or 150 mg/kg acrylonitrile and killed after 30 min. A rapid time- and dose-dependent decrease of GSH in the liver, lung, kidney and adrenals became apparent within 5 min of the injection. However even with a depletion of GSH of 80–90%, hepatic, renal and lung tissues showed no specific damage as revealed by gross or light-microscopic examination. The decrease in GSH concentrations in the liver and adrenals was gradual and steady, whereas, although minimal changes occurred at 10–50 mg acrylonitrile/kg, a sharp drop in cerebral GSH concentrations was observed between 50 and 150 mg/kg, the dose range that caused deaths.

In subacute experiments, acrylonitrile at concentrations of 0, 0·002, 0·01 or 0·05% was administered in the drinking-water over a 21-day period. Other animals received similar doses of acrylonitrile twice daily as a bolus administered by gavage. These experiments demonstrated a dose-dependent increase in hepatic GSH concentration, which was greater in the animals given a bolus dose. The authors point out that similar elevations have been demonstrated using chemical carcinogens, and ask the all-important question whether these changes will also reflect an early lesion preceding carcinogenic change in man.

NATURAL PRODUCTS

Taming tannic acid

Mitjavila, S., Lacombe, C., Carrera, G. & Derache, R. (1977). Tannic acid and oxidized tannic acid on the functional state of rat intestinal epithelium. *J. Nutr.* **107**, 2113.

Tannic acid (TA) in large doses has been shown to reduce the intestinal absorption of certain substances, such as glucose and methionine, possibly by forming insoluble complexes or by some direct action on the gut epithelium (*Cited in F.C.T.* 1972, **10**, 733). The paper cited above shows that the functional state of the intestinal epithelium of the rat is altered by feeding TA.

Male rats were fed diets containing 3% TA, 3% TA plus 1% sodium sulphite, or 3% oxidized TA, all calculated on the dry weight of the diet, for 25 days. Histological examination showed that animals on the first and third diets secreted a large amount of gastric mucus, although the quantity was not sufficient to afford complete protection; in all three groups, part of the superficial gastric mucosa was eroded, and this was accompanied by glandular atrophy in the group given TA alone. Mucus secretion in the duodenum was also increased, most markedly in the rats fed oxidized TA, and points of epithelial necrosis and zones of erosion were apparent. Epithelial cells isolated after 3 wk from the intestines of the rats of the first and last groups showed a reduced oxygen consumption and a significant decrease in succinic-dehydrogenase activity.

The faecal content of glucosamine, a constituent of mucous glycoproteins, was significantly increased by the feeding of TA, particularly in the unoxidized state. Excretion of sialic acids (constituents of mucin and mucopolysaccharides) was also increased by TA feeding. There was some disturbance of water and ionic balance in the faeces of TA-treated animals, a finding probably associated with altered mineral absorption.

In general simultaneous administration of an antioxidant, sodium sulphite, had little effect on the action of TA, and the degree of sulphite-TA interaction in the alimentary tract is difficult to assess. Oxidation prior to administration, however, reduced the damaging effect of TA on the gastric mucosa, and diminished the hypersecretion of mucus, thus reducing the loss of nitrogen in the faeces.

METHODS FOR ASSESSING TOXICITY

A prenatal development index

Courtney, K. D. (1977). Prenatal effects of herbicides: evaluation by the prenatal development index. *Archs env. contam. & Toxicol.* **6**, 33.

There is always a need to distinguish between teratogenicity, foetotoxicity and the foetoidal effects of compounds administered to the pregnant animal. However the evaluation of foetal development as a whole may be a useful concept, facilitating comparison of the overall toxicity of certain compounds on the developing foetus. The Prenatal Development Index has been proposed as a means of reducing the several toxicological parameters of the developing foetus to a single value.

The calculations involved for computing the index concern firstly the vehicle control data and then similar treatment of experimental data, thus:

Vehicle control

$$\frac{\text{Total number of normal live foetuses}}{\text{Total number of implantations}} = A'$$

Average foetal weight = B'

$A' \times B' = C$

Experimental

$$\frac{\text{Total number of normal live foetuses}}{\text{Total number of implantations}} = A$$

Average foetal weight = B

$A \times B = E$

$$\text{Prenatal Development Index} = \frac{E \times 100}{C}.$$

The validity of this index in toxicological evaluation was put to the test by comparing the foetal effects of herbicides of the phenoxyacetic acid class and related compounds. The herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and many of their esters produce cleft palate in mice. These compounds were administered to pregnant CD-1 mice by oral gavage or sc injection and the mice were killed on day 18 of gestation (day 1 being designated by the detection of a vaginal plug). Only live foetuses were examined for physical abnormalities, and foetuses were classified as abnormal if they had at least one anomaly regardless of type. Other foetal data required were the number of implantations, the number of live, normal foetuses, the percentage of foetal deaths and the average foetal weight. Maternal toxicity was indicated by measurement of maternal weight gain and the liver-to-body weight ratio. For most studies the vehicle was 0·1 ml corn oil–acetone (9:1). The index was calculated and compared over a range of experimental design (for example administration of the herbicide in solution or as a suspension), and the results were analysed statistically.

A direct comparison of the most commonly used herbicides using the Prenatal Development Index showed that 2,4-D, 2,4,5-T and Silvex (2,4,5-trichlorophenoxypropionic acid) adversely affected foetal development, the most active of the three being 2,4,5-T. The index of 2,4-D was unaffected by experimental design such as the vehicle and route of administration, but results with 2,4,5-T and Silvex depended upon the modes of administration. Silvex was in general less toxic but had maximal effect when given in dimethylsulphoxide.

Agent Orange, one of the defoliants used in Vietnam, is a 50–50 mixture of the *n*-butyl esters of 2,4-D and 2,4,5-T. It was found to be generally less active than the other herbicides in adversely affecting foetal development, and by comparison of its index with those of the constituent esters it was deduced that the two latter herbicides do not act by a similar mechanism.

This concept of viewing prenatal toxicity as a whole is an interesting one. The authors suggest that it will allow for the evaluation of compounds that result in a high incidence of foetal deaths and thus a low incidence of viable foetuses, and that would therefore be difficult to assess by other methods.

Fd Cosmet. Toxicol. Vol. 17, p. 183. Pergamon Press Ltd. 1979 Printed in Great Britain

MEETING ANNOUNCEMENT

SKIN MICROBIOLOGY: RELEVANCE TO CLINICAL INFECTION

An international symposium entitled "Skin Microbiology: Relevance to Clinical Infection" will be held in San Francisco on 29–31 August, 1979. The symposium will cover clinical infection in the fields of Dermatology, Surgery, Infectious Diseases, Paediatrics, etc. and will be open to members of all fields of medicine, nursing, and public health. Speakers will be experts from the United States and Western Europe. The course is sponsored by Extended Programs in Medical Education and presented by the Department of Dermatology of the University of California School of Medicine, San Francisco, California. Further information may be obtained from: Extended Programs in Medical Education, University of California, Room 569 U, San Francisco, CA 94143, USA (telephone no. (415) 666–4251).

ANALYSIS OF TRACE ORGANICS

An International Bioanalytical Forum on "Aids to Trace-organic Analysis" is being organized by the Wolfson Bioanalytical Centre of the University of Surrey and will be held at Guildford on 4–7 September 1979. Described as "an exchange of know-how amongst analysts with interests ranging from pollutants to drugs in blood", the Forum will be particularly concerned with the handling of environmental samples such as air, water, soils and foods, automatic procedures, analyses in biological samples, work-up procedures, and problems involved in setting up standards and establishing detection limits. The University's Institute of Industrial and Environmental Health and Safety is collaborating with the Centre on the environmental aspects of the Forum. Further information may be obtained from Dr. E. Reid, Wolfson Bioanalytical Centre, University of Surrey, Guildford, GU2 5XH (telephone no. 0483 71281).

Fd Cosmet. Toxicol. Vol. 17, p. 185. Pergamon Press Ltd. 1979. Printed in Great Britain

FORTHCOMING PAPERS

It is hoped to publish the following research papers and other communications in the next issue of *Food and Cosmetics Toxicology*:

Long-term toxicity studies on coumarin in the baboon. By J. G. Evans, I. F. Gaunt and B. G. Lake.

Response of male rats to sodium saccharin ingestion: urine composition and mineral balance. By R. L. Anderson.

Intestinal absorption of two potential polymeric food additives in man. By P. D. Walson, D. E. Carter, B. A. Ryerson, S. C. Halladay and T. M. Parkinson.

Results of a two-year chronic toxicity and oncogenic study of rats ingesting diets containing 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). By R. J. Kociba, D. G. Keyes, R. W. Lisowe, R. P. Kalnins, D. D. Dittenber, C. E. Wade, S. J. Gorzinski, N. H. Mahle and B. A. Schwetz.

Analysis of faeces for benzo[*a*]pyrene after consumption of charcoal-broiled beef by rats and humans. By S. S. Hecht, W. Grabowski and K. Groth.

Absence of mutagenic activity in *Salmonella typhimurium* of some impurities found in saccharin. By F. Poncelet, M. Roberfroid, M. Mercier and J. Lederer. (Short Paper)

Toxicity of *Diplodia macrospora* to laboratory animals. By N. P. J. Kriek and W. F. O. Marasas. (Short Paper)

Dihydroaflatoxin inhibition of energy-linked reduction of endogenous nicotinamide nucleotides in isolated rat-liver mitochondria. By O. Obidoa and E. E. Obonna. (Short Paper)

In vitro inhibition of rat intestinal histamine-metabolizing enzymes. By S. L. Taylor and E. R. Lieber. (Short Paper)

Critical review of the toxicology of coumarin with special reference to interspecies differences in metabolism and hepatotoxic response and their significance to man. By A. J. Cohen. (Review Paper)

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[*Contents continued*]

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